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<b>(21) International Application Number:</b> PCT/US96/14154 <b>(22) International Filing Date:</b> 22 August 1996 (22.08.96)  <b>(30) Priority Data:</b> 60/002,615      22 August 1995 (22.08.95)      US Not furnished      21 August 1996 (21.08.96)      US  <b>(71) Applicant:</b> THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).  <b>(72) Inventors:</b> SCHNEEWIND, Olaf; 11050 Strathmore Drive #422, Los Angeles, CA 90024 (US). BABA, Tadashi; 945 Gayley #306, Los Angeles, CA 90024 (US).  <b>(74) Agent:</b> FARBER, Michael, B.; Merchant, Gould, Smith, Edell, Welter & Schmidt, Suite 400, 11150 Santa Monica Boulevard, Los Angeles, CA 90025 (US).		<b>(81) Designated States:</b> AU, BR, CA, IL, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> TARGETING OF PROTEINS TO THE CELL WALL OF GRAM-POSITIVE BACTERIA		
<b>(57) Abstract</b> <p>A method of stable noncovalent display of proteins, peptides, or compounds covalently linked to proteins or peptides on the surface of Gram-positive bacteria provides advantages over phage display. One embodiment of the present invention comprises a method for noncovalent protein targeting, comprising the steps of: (1) cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal (2) growing the bacterium into which the nucleic acid segment has been cloned to express the chimeric protein to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal; and (3) binding the expressed chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface on the Gram-positive bacterium in such a way that the protein is accessible to a ligand. Alternatively, the chimeric protein can be produced by expression in another expression system and contacted with the Gram-positive bacterium.</p>		

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TARGETING OF PROTEINS TO THE CELL WALL OF  
GRAM-POSITIVE BACTERIA

GOVERNMENT RIGHTS

This invention was supported by grants from the United States Government, namely grants from the National Institutes of Health. Accordingly, the government may  
5 have certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention is directed to methods for targeting of proteins to the cell wall of Gram-positive  
10 bacteria, particularly *Staphylococcus*, and the use of such targeted proteins.

For many purposes, it is desirable to display proteins or peptides on the surfaces of bacteria so that the proteins or peptides are accessible to the  
15 surrounding solution and can, for example, be bound by a ligand that is bound specifically by the protein or peptide. In particular, the display of proteins on the surface of bacteria is desirable for the preparation of vaccines, the linkage of molecules such as antibiotic  
20 molecules or diagnostic reagents to cells, in screening reagents such as monoclonal antibodies, and in the selection of cloned proteins by displaying the cloned proteins and then observing their reaction with specific reagents such as antibodies. One way of doing this has  
25 been with phage display (G.P. Smith, "Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface," Science 228:1315-1316 (1985)). However, phage display is limited in its

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practicality, because it requires that the protein to be displayed be inserted into a coat protein of filamentous phage and retain its activity while not distorting the conformation of the coat protein, allowing functional virions to be formed. In general, this technique is therefore limited only to small peptides and proteins.

Therefore, there is a need for a more general method of peptide and protein display. Preferably such a method is capable of displaying proteins that have been expressed not only in Gram-positive bacteria such as *Staphylococcus aureus*, but in Gram-negative bacteria such as *Escherichia coli*, *Salmonella typhimurium* and other Gram-negative bacteria. Preferably, such a method is also capable of binding proteins that have been expressed in eukaryotic cells such as insect or animal cells.

#### SUMMARY

We have developed a method of stable noncovalent display of proteins, peptides, or compounds covalently linked to proteins or peptides on the surface of Gram-positive bacteria, particularly *Staphylococcus aureus* and other species of *Staphylococcus*.

One embodiment of the present invention comprises a method for protein targeting and display employing expression of a chimeric protein in a Gram-positive bacterium and subsequent targeting of the chimeric protein to the cell wall and display of that protein. In general, such a method comprises the steps of:

(1) cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to

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generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal;

(2) growing the bacterium into which the nucleic acid segment has been cloned to express the cloned chimeric protein in the Gram-positive bacterium to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal;

(3) binding the expressed chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

Typically, the carboxyl-terminal cell-wall targeting signal is any of: (a) the lysostaphin signal having an amino acid sequence of W-K-T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-W-V-G-Y-Y-G-N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-L-W-G-T-I-K (SEQ ID NO: 1); (b) the amidase signal having an amino acid sequence of V-A-S-A-W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2); (c) a signal related to the lysostaphin signal or the amidase signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal, including signals from other Gram-positive bacteria; and (d) a signal related to these signals by truncation.

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The carboxyl-terminal cell-wall targeting signal can be a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions  
 5 occurring in both the lysostaphin signal and the amidase signal.

The carboxyl-terminal cell-wall targeting signal can have the sequence W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-  
 10 X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>,  
 wherein the residues designated by subscripts are chosen so that the conformation of the targeting signal remains  
 15 substantially equivalent to that of the lysostaphin or amidase targeting signals.

In this sequence, X<sub>3</sub> can be R or T, X<sub>9</sub> is Y or L, X<sub>11</sub> is M or K, X<sub>12</sub> is E or S, X<sub>16</sub> is R or S, X<sub>19</sub> is N or P, X<sub>20</sub> is G or N, X<sub>21</sub> is N or T, X<sub>22</sub> is Q or D, X<sub>23</sub> is P or  
 20 I, X<sub>26</sub> is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X<sub>28</sub> is K or T, X<sub>29</sub> is V or T, X<sub>33</sub> is L or R, X<sub>35</sub> is G or M, X<sub>37</sub> is V or Q, X<sub>38</sub> is G or S, X<sub>39</sub> is Y or G, X<sub>40</sub> is Q or V, X<sub>41</sub> is F or L, X<sub>42</sub> is Q or K, X<sub>43</sub> is P or A, X<sub>45</sub> is G  
 25 or Q, X<sub>46</sub> is Y or T, X<sub>47</sub> is C or I, X<sub>48</sub> is D or H, X<sub>50</sub> is T or D, X<sub>54</sub> is L or K, X<sub>65</sub> is W or G, X<sub>66</sub> is E or N, X<sub>67</sub> is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X<sub>71</sub> is Y or I, X<sub>80</sub> is G or K, X<sub>82</sub> is A or T, X<sub>83</sub> is P or N, X<sub>84</sub>  
 30 is P or T, X<sub>85</sub> is N or an absent amino acid with an amide bond between the preceding and following amino acids in

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the chain,  $X_{86}$  is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{87}$  is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{94}$  is E or T, and  $X_{96}$  is S or K.

The conservative amino acid substitutions in highly conserved regions can be the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa. The conservative amino acid substitutions in less highly conserved regions can be the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

Alternatively, the carboxyl-terminal cell wall targeting signal can have from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

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As an alternative to expression in a Gram-positive bacterium and stable noncovalent binding to the cells in which the chimeric protein are expressed, the targeting method can comprise:

5           (1) generating a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal by expression of a nucleotide sequence encoding the chimeric protein in an expression system other than an expression system employing a Gram-positive bacterium as host;

10           (2) adding the chimeric protein to a culture medium in which a Gram-positive bacterium is growing; and

            (3) allowing the chimeric protein to bind stably and noncovalently to the cell wall of the Gram-positive bacterium so that the chimeric protein is  
15 displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand..

Another aspect of the present invention is a method for producing a vaccine to an antigen comprising  
20 immunizing an antibody-producing animal with a complex comprising a chimeric protein including therein: (1) a protein antigen and (2) a carboxyl-terminal cell wall-targeting signal bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-  
25 terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the chimeric protein is accessible to the antigen-processing system of the antibody-producing animal.

30           The antigen can be a polypeptide antigen produced by *Candida albicans*, *Aspergillus fumigatus*,



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*Histoplasma capsulatum*, *Microsporium canis*, *Plasmodium falciparum*, *Trypanosoma cruzi*, *Borrelia bergdorferi*, *Treponema pallidum*, *Borrelia recurrentis*, *Leptospira icterohaemorrhagiae*, *Neisseria gonorrhoeae*,  
5 *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhosa*, *Hemophilus influenzae*, *Bordetella pertussis*, *Actinomyces israelii*, *Streptococcus mutans*, *Streptococcus equi*, *Streptococcus agalactiae*, *Streptococcus anginosus*, human immunodeficiency virus (HIV), poliovirus, influenza  
10 virus, rabies virus, herpes virus, foot and mouth disease virus, psittacosis virus, paramyxovirus, myxovirus, or coronavirus.

Alternatively, the antibody-producing animal can be immunized with a complex comprising a non-protein  
15 antigen or hapten covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal for production of vaccines. The antigen or hapten can be a drug, an alkaloid, a steroid, a carbohydrate, or an aromatic compound. The same method can be used for  
20 vaccination with a protein antigen, by immunizing an antibody-producing animal with a complex comprising a protein antigen covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal.

Another aspect of the present invention is a  
25 method for screening a cloned protein for reactivity with a specific binding partner comprising:

(1) incorporating a cloned protein into a chimeric protein including therein a carboxyl-terminal cell wall-targeting signal;

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(2) binding the chimeric protein to the surface of a Gram-positive bacterium to form a stable noncovalent complex; and

(3) reacting the noncovalent complex with a  
5 labeled specific binding partner to screen the cloned protein for reactivity with the specific binding partner.

The labeled specific binding partner is typically an antibody.

Another aspect of the present invention is a  
10 method for treating an infection caused by a Gram-positive bacterium comprising:

(1) conjugating an antibiotic to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate; and

15 (2) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the conjugate noncovalently to the cell walls of the bacterium in order to treat the infection.

An analogous method can be used to detect the  
20 presence of a Gram-positive bacterium and thereby diagnose an infection by conjugating a diagnostic reagent to the protein instead of the antibiotic and binding the conjugate to the cell walls of the bacterium.

Another aspect of the present invention is a  
25 chimeric protein comprising:

(1) a protein to be targeted to the cell wall of a Gram-positive bacterium; and

(2) a cell-wall targeting signal located at the carboxyl terminus of the chimeric protein for binding  
30 the chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting

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signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

Additional aspects of the present invention

5 include DNA or other nucleic acid segments encoding such chimeric proteins; vectors incorporating the DNA or other nucleic acid segments encoding the chimeric proteins; stable noncovalent complexes of bacterial cells and cell-wall targeted proteins; covalent conjugates of protein or

10 non-protein antigens and cell-wall targeted peptides, optionally including a spacer; and noncovalent complexes of these conjugates and Gram-positive bacteria.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and accompanying drawings where:

Figure 1A shows the structures of wild-type

20 lysostaphin (LST) and its mutants;

Figure 1B shows pulse-chase experiments to determine the localization of lysostaphin in subcellular fractions after pulse-labeling with [<sup>35</sup>S] methionine followed by a chase of non-radioactive methionine; pulse-

25 labeled lysostaphin was immunoprecipitated with a specific antiserum, separated on a polyacrylamide gel and fluorographed;

Figure 1C is a similar experiment showing that secreted LST from *Staphylococcus simulans* cells was not

30 cleaved rapidly even in the presence of *S. aureus* cells;

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Figure 2A shows the structures of staphylococcal enterotoxin B (SEB) and its hybrid proteins with: (1) a C-terminal fusion of the cell wall sorting signal of staphylococcal protein A which consists of the LPXTG motif, the C-terminal hydrophobic domain and the charged tail; (2) the membrane anchor segment of *Listeria monocytogenes* ActA; (3) the cell-wall targeting domain (C-terminal 92 amino acids) of lysostaphin; (4) and the cell-wall targeting domain (C-terminal 99 amino acids) of staphylococcal amidase;

Figure 2B shows a pulse-chase experiment in *S. aureus* OS2 expressing genes of various hybrid proteins, showing the localization of the resulting proteins in various cell components or in the culture medium;

Figure 2C shows a similar experiment in which the samples were digested with either lysostaphin (L) or Chalaropsis B muramidase (Hash-enzyme) (H) and precipitated; proteins were solubilized in hot SDS, immunoprecipitated with anti-SEB, and subjected to 12% SDS-polyacrylamide gel electrophoresis;

Figure 3 shows the surface display of proteins targeted to the cell wall of *S. aureus*; staphylococci expressing wild-type or mutant protein A were harvested by centrifugation, washed and incubated with FITC-labeled rabbit immunoglobulin (FITC-IgG); binding of FITC-IgG to protein located on the staphylococcal surface was visualized by microscopy under ultraviolet light; panel (1) is cells expressing wild-type protein A; panel (2) is cells expressing protein A with a C-terminal cell-wall targeting signal; and panel (3) is cells expressing a mutant protein A (SPA<sub>1-519</sub>) known to be secreted into the

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culture medium; the presence of staphylococci in this panel is demonstrated by light microscopy (3 visible light);

Figure 4 is a diagram showing the homology  
5 between the amidase and lysostaphin amino acid sequences;

Figure 5A demonstrates the targeting of a hybrid glutathione S-transferase to *S. aureus*; the glutathione S-transferase activity remaining in the supernatant after reaction with added *S. aureus* (squares)  
10 or *S. simulans* (circles) cells is shown;

Figure 5B shows the same result by polyacrylamide gel electrophoresis, showing increasing binding of the hybrid glutathione S-transferase to *S. aureus*, but not to *S. simulans*, as shown by increasing  
15 presence of the hybrid protein in the cell pellet for *S. aureus* but not for *S. simulans*;

Figure 6 is a table showing the target cell specificity and bacteriolytic activity of mature wild-type lysostaphin, pro-lysostaphin, and a mutant of  
20 lysostaphin in which a portion of the cell wall targeting signal is deleted;

Figure 7A shows the structures of wild-type lysostaphin (LST, 1) and hybrid lysostaphins with a C-terminal fusion of (2) the cell wall sorting signal of protein A; (3) a sorting signal devoid of its LPXTG (SEQ  
25 ID NO: 10) motif, as well as hybrid enterotoxin B molecules with: (4) a C-terminal fusion of both the lysostaphin targeting signal and the protein A targeting signal or (5) a similar molecule without a LPXTG (SEQ ID  
30 NO: 10) motif; and

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Figure 7B shows the results of polyacrylamide gel electrophoresis and fluorography to analyze the cell wall linkage of the hybrid proteins.

5    DESCRIPTION

We have developed a method for the stable and noncovalent targeting of proteins to the cell wall of Gram-positive bacteria, particularly *Staphylococcus aureus*.

10       Proteins targeted to the cell wall are accessible to the surrounding medium and retain their ability to bind ligands, such as substrates, inhibitors, allosteric modulators, hormones, ligands, antigens, haptens, or small molecules such as metal ions.

15

I.   PROTEIN TARGETING BY EXPRESSION IN GRAM-POSITIVE BACTERIA

One aspect of the present invention is a method for protein targeting and display employing expression of a chimeric protein in a Gram-positive bacterium and subsequent targeting of the chimeric protein to the cell wall and display of that protein. In general, such a method comprises the steps of:

25       (1) Cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal that is any of: (a) the lysostaphin signal; (b) the amidase signal; (c) a signal related to the lysostaphin signal or the  
30   amidase signal by one or more conservative amino acid substitutions that preserve the existence of consensus

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sequence regions occurring in both the lysostaphin signal and the amidase signal, including signals from other Gram-positive bacteria; and (d) a signal related to these signals by truncation.

5           (2) Growing the bacterium into which the nucleic acid segment has been cloned to express the cloned chimeric protein to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal.

10           (3) Stable noncovalent binding of the expressed chimeric protein to the cell wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface on the Gram-positive bacterium in such a way that the protein is  
15 accessible to a ligand.

As used herein, the terms "noncovalent" and "noncovalently" refer to the initial binding between the expressed chimeric protein and the bacterial cell wall and do not exclude subsequent covalent fixation of the  
20 chimeric protein to the cell wall by the use of crosslinking reagents such as glutaraldehyde, the formation of Schiff bases that are stabilized by reduction with borohydride, or other methods known in the art.

25           Typically, the Gram-positive bacterium is a species of *Staphylococcus*. A particularly preferred species of *Staphylococcus* is *Staphylococcus aureus*. However, other Gram-positive bacteria such as  
30 *Streptococcus pyogenes*, other *Streptococcus* species, and Gram-positive bacteria of other genera can also be used.

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A. Cloning the Nucleic Acid Segment Into the Gram-Positive Bacterium

Cloning the nucleic acid segment encoding the chimeric protein into the Gram-positive bacterium is performed by standard methods. In general, such cloning involves: (1) isolation of a nucleic acid segment encoding the protein to be targeted; (2) joining to the cell-wall targeting signal to produce a chimeric nucleic acid segment encoding both the protein to be targeted and the cell-wall targeting signal; (3) cloning by insertion into a vector compatible with the Gram-positive bacterium in which expression is to take place; and (4) incorporation of the vector including the chimeric nucleic acid segment into the bacterium.

Typically, the nucleic acid segment encoding the protein to be targeted is DNA; however, the use of RNA in certain cloning steps is within the scope of the present invention. As used herein, the term "nucleic acid segment" includes both DNA and RNA unless otherwise specified, and, unless otherwise specified, includes both double-stranded and single-stranded nucleic acids. Also included are hybrids such as DNA-RNA hybrids. In particular, a reference to DNA includes RNA that has either the equivalent base sequence except for the substitution of uracil in RNA for thymine in DNA, or has a complementary base sequence except for the substitution of uracil for thymine, complementarity being determined according to the Watson-Crick base pairing rules. Reference to a nucleic acid sequence can also include modified bases as long as the modification does not significantly interfere either with binding of a ligand



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such as a protein by the nucleic acid or Watson-Crick base pairing.

When dealing with genes from eukaryotic organisms, it is preferred to use cDNA, because the

5 natural gene typically contains intervening sequences or introns that are not translated. Alternatively, if the amino acid sequence is known, a synthetic gene encoding the protein to be targeted can be constructed by standard solid-phase oligodeoxyribonucleotide synthesis methods,

10 such as the phosphotriester or phosphite triester methods. The sequence of the synthetic gene is determined by the genetic code, by which each naturally-occurring amino acid is specified by one or more triplet codons. Additionally, if a portion of the protein

15 sequence is known, but the gene or messenger RNA has not been isolated, the amino acid sequence can be used to construct a degenerate set of probes according to the known degeneracy of the genetic code. General aspects of cloning are described, for example, in J. Sambrook et

20 al., "Molecular Cloning: A Laboratory Manual" (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989); in B. Perbal, "A Practical Guide to Molecular Cloning" (2d ed., John Wiley & Sons, New York 1988), in S.L. Berger & A.R. Kimmel, "Guide to Molecular

25 Cloning Techniques" (Methods in Enzymology, vol. 152, Academic Press, Inc., San Diego, 1987), and in D.V. Goeddel, ed., "Gene Expression Technology" (Methods in Enzymology, vol. 185, Academic Press, Inc., San Diego, 1991).

30 Once isolated, DNA encoding the protein to be targeted is then joined to the cell-wall targeting

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signal. This is typically accomplished through ligation, such as using *Escherichia coli* or bacteriophage T4 ligase. Conditions for the use of these enzymes are well known and are described, for example, in the above  
5 general references.

The ligation is done in such a way so that the targeted protein and the cell-wall targeting signal are joined in a single contiguous reading frame so that a single protein is produced. This may, in some cases,  
10 involve addition or deletion of bases to the cloned DNA segment to maintain a single reading frame. This can be done using standard techniques.

Cloning is typically performed by inserting the cloned DNA into a vector containing control elements to  
15 allow expression of the cloned DNA. The vector is then incorporated into the bacterium in which expression is to occur, using standard techniques of transformation or other techniques for introducing nucleic acids into bacteria.

20 One suitable cloning system for *S. aureus* places the cloned gene under the control of the BlaZRI regulon (P.Z. Wang et al., Nucl. Acids Res. 19:4000 (1991)). Vectors and other cloning techniques for use in *Staphylococcus aureus* are described in B. Nilsson & L. Abrahmsen, "Fusion to Staphylococcal Protein A," in Gene Expression Technology, supra, pp. 144-161.  
25

If the chimeric protein is cloned under control of the BlaZRI regulon, expression can be induced by the addition of the  $\beta$ -lactam antibiotic methicillin.

30 The cell-wall targeting signal that can be used can be any of: (1) the lysostaphin signal; (2) the

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amidase signal; (3) a signal related to the lysostaphin signal or the amidase signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the  
5 lysostaphin signal and the amidase signal, including signals from other Gram-positive bacteria; and (4) a signal related to the lysostaphin signal or the amidase signal by truncation.

10 1. The Lysostaphin Signal

The lysostaphin signal comprises the 92 carboxyl-terminal residues in lysostaphin, a bacteriolytic enzyme that cleaves the pentaglycine crossbridges of staphylococcal peptidoglycans (C.A. Schindler & V.T. Schuhardt, Proc. Natl. Acad. Sci. USA  
15 51:414 (1964)).

This sequence comprises the C-terminal 92 amino acids of lysostaphin (LST), namely amino acid residues 155-246 (P.A. Recsei et al., "Cloning, Sequence, and  
20 Expression of the Lysostaphin Gene From *Staphylococcus simulans*," Proc. Natl. Acad. Sci. USA 84:1127-1131 (1987)). This sequence is: W-K-T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-W-V-G-Y-Y-G-N-S-G-Q-R-I-  
25 Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-L-W-G-T-I-K (SEQ ID NO: 1).

Identical signals are known to exist in other Gram-positive organisms. For example, the bacterium *Staphylococcus staphylolyticus* has a carboxyl-terminal  
30 region of its lysostaphin protein, residues 389-480, that is identical with the carboxyl-terminal residues of the

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lysostaphin protein in *S. simulans* that include the C-terminal cell-wall targeting signal.

## 2. The Amidase Signal

5 Another preferred sequence according to the present invention is the carboxyl-terminal 99 amino acids of staphylococcal amidase (LytA) (A. Wang et al., "Sequence Analysis of a *Staphylococcus aureus* Gene Encoding a Peptidoglycan Hydrolase Activity," Gene 10 102:105-109 (1991)). This sequence is: V-A-S-A-W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

15

## 3. Signals Related to the Lysostaphin Signal or the Amidase Signal by One or More Conservative Amino Acid Substitutions

Other C-terminal cell-wall targeting sequences 20 exist that are suitable for use in chimeric proteins for processes according to the present invention.

For example, there is a substantial consensus between the C-terminal cell-wall targeting sequences of the amidase and lysostaphin gene. If the conserved 25 residues are taken into account, most generally, the consensus sequence is: W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>.

30

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Although the residues referred to by subscripts can be varied broadly, as discussed below, as long as the conformation of the resulting sequence is not altered significantly, preferably, within this consensus

5 sequence,  $X_3$  is selected from the group consisting of R and T,  $X_9$  is selected from the group consisting of Y or L,  $X_{11}$  is selected from the group consisting of M and K,  $X_{12}$  is selected from the group consisting of E and S,  $X_{16}$  is selected from the group consisting of R and S,  $X_{19}$  is  
10 selected from the group consisting of N and P,  $X_{20}$  is selected from the group consisting of G and N,  $X_{21}$  is selected from the group consisting of N or T,  $X_{22}$  is selected from the group consisting of Q and D,  $X_{23}$  is selected from the group consisting of P and I,  $X_{26}$  is  
15 selected from the group consisting of V and an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{28}$  is selected from the group consisting of K and T,  $X_{29}$  is selected from the group consisting of V and T,  $X_{33}$  is selected from the  
20 group consisting of L and R,  $X_{35}$  is selected from the group consisting of G and M,  $X_{37}$  is selected from the group consisting of V and Q,  $X_{38}$  is selected from the group consisting of G and S,  $X_{39}$  is selected from the group consisting of Y and G,  $X_{40}$  is selected from the  
25 group consisting of Q and V,  $X_{41}$  is selected from the group consisting of F and L,  $X_{42}$  is selected from the group consisting of Q and K,  $X_{43}$  is selected from the group consisting of P and A,  $X_{45}$  is selected from the group consisting of G and Q,  $X_{46}$  is selected from the  
30 group consisting of Y and T,  $X_{47}$  is selected from the group consisting of C and I,  $X_{48}$  is selected from the

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group consisting of D and H,  $X_{50}$  is selected from the  
group consisting of T and D,  $X_{54}$  is selected from the  
group consisting of L and K,  $X_{65}$  is selected from the  
group consisting of W and G,  $X_{66}$  is selected from the  
5 group consisting of E and N,  $X_{67}$  is selected from the  
group consisting of an absent amino acid with an amide  
bond between the preceding and following amino acids in  
the chain and S,  $X_{71}$  is selected from the group consisting  
of Y and I,  $X_{80}$  is selected from the group consisting of G  
10 and K,  $X_{82}$  is selected from the group consisting of A and  
T,  $X_{83}$  is selected from the group consisting of P and N,  
 $X_{84}$  is selected from the group consisting of P and T,  $X_{85}$   
is selected from the group consisting of N and an absent  
amino acid with an amide bond between the preceding and  
15 following amino acids in the chain,  $X_{86}$  is selected from  
the group consisting of Q and an absent amino acid with  
an amide bond between the preceding and following amino  
acids in the chain,  $X_{87}$  is selected from the group  
consisting of I and an absent amino acid with an amide  
20 bond between the preceding and following amino acids in  
the chain,  $X_{94}$  is selected from the group consisting of E  
and T, and  $X_{96}$  is selected from the group consisting of S  
and K.

This sequence can be extended by additional  
25 residues at the amino-terminus, that is the terminus  
immediately adjacent to the carboxyl-terminus of the  
targeted protein in the fusion construct.

For the purposes of this discussion, this  
consensus sequence can be divided into completely  
30 conserved regions, in which the sequences for lysostaphin  
and amidase agree in best alignment, and less highly

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conserved regions, in which the sequences diverge. This distinction, as used here, is not intended to imply a functional difference.

In the less highly conserved portions of the  
5 consensus sequence, conservative amino acid substitutions  
can be made according to general principles of protein  
chemistry. Such conservative amino acid substitutions  
include, but are not limited to, substituting any of  
isoleucine (I), valine (V), and leucine (L) for any other  
10 of these amino acids; aspartic acid (D) for glutamic acid  
(E) and vice versa; glutamine (Q) for asparagine (N) and  
vice versa, and serine (S) for threonine (T) and vice  
versa. The above-mentioned substitutions are not the  
only amino acid substitutions that can be considered  
15 "conservative." Other substitutions can also be  
considered conservative, depending upon the environment  
of the particular amino acid. For example, glycine (G)  
and alanine (A) can frequently be interchangeable, as can  
be alanine and valine (V). Methionine (M), which is  
20 relatively hydrophobic, can frequently be interchanged  
with leucine and isoleucine, and sometimes with valine.  
Lysine (K) and arginine (R) are frequently  
interchangeable in locations in which the significant  
feature of the amino acid residue is its charge and the  
25 different pK's of these two amino acid residues or their  
different sizes are not significant. Still other changes  
can be considered "conservative" in particular  
environments. For example, if an amino acid on the  
surface of a protein is not involved in a hydrogen bond  
30 or salt bridge interaction with another molecule, such as  
another protein subunit or a ligand bound by the protein,

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negatively charged amino acids such as glutamic acid and aspartic acid can be substituted for positively charged amino acids such as lysine or arginine and vice versa. Histidine (H), which is more weakly basic than arginine  
5 or lysine, and is partially charged at neutral pH, can sometimes be substituted for these more basic amino acids. Additionally, the amides glutamine and asparagine can sometimes be substituted for their carboxylic acid homologues, glutamic acid and aspartic acid.

10 In the completely conserved regions of the consensus sequence, it is likely that highly conservative amino acid substitutions, such as any of isoleucine, valine, and leucine for any of these other amino acids, aspartic acid for glutamic acid and vice versa, glutamine  
15 for asparagine and vice versa, and serine for threonine and vice versa are allowable substitutions; other substitutions may be allowable if the conformation of the protein remains substantially unchanged.

The fact that certain amino acids can be  
20 omitted in this consensus sequence means that the total length of the consensus sequence can vary. In fact, a total of five amino acids can be omitted from the sequence, leaving a total of 91. Clearly, any intermediate number of amino acids between these limits,  
25 i.e., between 91 and 96, can be tolerated within the consensus sequence. Thus, any sequence of between 91 and 96 amino acid residues that has only highly conservative amino acid substitutions in the completely conserved regions as determined by a comparison of the LST and LytA  
30 sequences, and has the somewhat less highly conservative amino substitutions described above in the less highly



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conservative regions, is suitable for cell-wall targeting according to the methods of the present invention.

Still other partially homologous sequences are expected to have cell-wall targeting activity. These  
5 include a sequence from the F or S component of the Pantan-Valentine leucocidins produced by *Staphylococcus aureus*. These components include a sequence at residues 89-136 of T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID  
10 NO: 3), substantially homologous to a portion of the lysostaphin sequence. There are 18 out of 48 exact matches, and 26 out of 48 exact matches or homologous amino acids.

Another protein expected to have cell-wall  
15 targeting activity is a cell-wall protein precursor in *Staphylococcus mutans*, strain OMZ175, serotype f. This sequence includes a sequence of S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4). This sequence has 37% exact matches with  
20 the lysostaphin sequence, and 55% exact matches or homologous amino acids. Cell-wall targeting sequences useful in the methods of the present invention can therefore include amino acid segments including these sequences, with a total length of about 80 to 100 amino  
25 acids.

Because signals according to the present invention are intended to be operable as part of longer chimeric fusion proteins, it is believed that these signals remain functional even when additional amino acid  
30 residues are appended to their carboxyl termini, or when

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additional amino acid residues are appended to the amino termini of the targeted proteins.

#### 4. Truncated Signals

5           Comparison of the signals recited above indicates that conserved regions exist throughout the signal sequence and that no single conserved region is required to obtain a functional signal. This suggests that some portions of the signal sequence can be omitted  
10 without abolishing the binding to the cell surface. Therefore, signals that are related to the above-described signals by truncation in such a way that the total length of the signal sequence is reduced to no less than about 80 amino acids and the best alignment of the  
15 truncated signal and the original signal results in at least about 80% homology and at least about 70% identity are also useful in processes according to the present invention.

#### 20           B. Expression in the Gram-Positive Bacterium to Generate a Chimeric Protein Including Therein a C-Terminal Cell-Wall Targeting Signal

          The next step is expression in a Gram-positive bacterium to generate a chimeric protein including  
25 therein a C-terminal cell-wall targeting signal. Expression is typically under the control of various control elements associated with the vector incorporating the DNA encoding the chimeric protein; such elements can include promoters and operators, which can be regulated  
30 by proteins such as repressors. The conditions required for expression of cloned proteins in Gram-positive

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bacteria, particularly *S. aureus*, are well known in the art and need not be further recited here. An example is the induction of expression of the lysostaphin gene under control of the BlaZRI regulon induced by the addition of methicillin.

When expressed in *Staphylococcus aureus*, the chimeric protein is first exported with an amino-terminal leader peptide, such as the hydrophobic region of Figure 1 or the hydrophobic signal peptide at the amino-terminal region of the cloned lysostaphin of Recsei et al. (P. Recsei et al., "Cloning, Sequence, and Expression of the Lysostaphin Gene from *Staphylococcus simulans*," Proc. Natl. Acad. Sci. USA 84: 1127-1131 (1987)).

#### 15 C. Stable Noncovalent Binding of the Chimeric Proteins to the Cell Wall

The third step is stable noncovalent binding of the chimeric protein to the cell wall via the C-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that it is accessible to a ligand. This binding is noncovalent, as shown in Example 1. However, it is stable under conditions in which noncovalent complexes such as antigen-antibody or hapten-antibody complexes are stable, allowing the use of a complex such as in immunization for the production of vaccines.

#### 30 II. CLONING AND EXPRESSION IN CELLS OTHER THAN GRAM-POSITIVE BACTERIA AND SUBSEQUENT TARGETING OF CHIMERIC PROTEINS TO THE CELL WALL OF GRAM-POSITIVE BACTERIA

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As an alternative to the cloning and expression of a chimeric protein containing the cell-wall targeting signal in Gram-positive bacteria such as *S. aureus* and the subsequent binding of the chimeric protein to the cell wall of the bacteria in which the chimeric protein was expressed, the chimeric protein can be generated by expression in another expression system, added to the culture medium of the Gram-positive bacteria such as *S. aureus*, and allowed to bind noncovalently to the cell wall. In other words, even if the chimeric protein is produced from another source, the cell-wall targeting signal of the chimeric protein directs the molecules to the cell wall of Gram-positive bacteria such as *S. aureus* to form a stable noncovalent association.

For this embodiment of a method according to the present invention, the assembly of the chimeric protein by genetic engineering techniques is basically same as discussed above in section I(A), and the cell-wall targeting signals used can be the same. In this system, expression can be in any expression system commonly used in the art such as described in the references by Sambrook et al., Perbal, Berger & Kimmel, and Goeddel. In general, these methods require the insertion of the DNA encoding the chimeric protein including the C-terminal cell-wall targeting signal into a suitable vector. This vector is then introduced into the cell. Suitable vectors and methods for incorporating the DNA encoding the chimeric protein into the vectors and introducing the vectors into the cells used for expression are described in the above-identified references and elsewhere. Among the expression systems

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that can be used are Gram-negative bacteria such as *E. coli* and *S. typhimurium*, *Bacillus subtilis*, mammalian cells, and insect cells. The vectors required for these different cell types are well known in the art and need  
5 not be further described here; an example is the use of baculovirus vectors for insect cells.

The proteins resulting from expression, which typically are secreted into the culture medium in the cells producing them, are then isolated, purified if  
10 desired, and contacted with the Gram-positive bacteria such as *S. aureus* to allow the chimeric proteins containing the C-terminal cell-wall targeting signal to bind to the cell wall of the Gram-positive bacteria. This contact normally occurs in conditions compatible  
15 with the growth of the Gram-positive bacteria.

This embodiment greatly expands the utility of the method by allowing cloning and expression in any organism that will support an expression system.

20 III. USE OF CELL-WALL TARGETED PROTEINS AND COMPLEXES BETWEEN CELL-WALL TARGETED PROTEINS AND GRAM-POSITIVE BACTERIA

Cell-wall targeted proteins and complexes between cell-wall targeted proteins and Gram-positive  
25 bacteria according to the present invention have a number of uses.

One use is use in the production of vaccines that can be used to generate immunity against infectious diseases affecting mammals, including both humans and  
30 non-human mammals, such as cattle, sheep, goats, poultry, and fish. This invention is of special importance to

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mammals. The usefulness of these complexes for vaccine production lies in the fact that the proteins are on the surface of the cell wall and are accessible to the medium surrounding the bacterial cells, so that the antigenic  
5 part of the chimeric protein is accessible to the antigen processing system. It is well known that presenting antigens in particulate form greatly enhances the immune response. In effect, bacteria containing antigenic peptides on their surfaces linked to the bacteria by  
10 these noncovalent but stable interactions function as natural adjuvants. Here follows a representative list of typical microorganisms that express polypeptide antigens against which useful antibodies can be prepared by the methods of the present invention:

15 (1) Fungi: *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum* (all cause disseminating disease), *Microsporium canis* (animal ringworm).

20 (2) Parasitic protozoa: (1) *Plasmodium falciparum* (malaria), *Trypanosoma cruzi* (sleeping sickness).

25 (3) Spirochetes: (1) *Borrelia bergdorferi* (Lyme disease), *Treponema pallidum* (syphilis), *Borrelia recurrentis* (relapsing fever), *Leptospira icterohaemorrhagiae* (leptospirosis).

30 (4) Bacteria: *Neisseria gonorrhoeae* (gonorrhea), *Staphylococcus aureus* (endocarditis), *Streptococcus pyogenes* (rheumatic fever), *Salmonella typhosa* (salmonellosis), *Hemophilus influenzae* (influenza), *Bordetella pertussis* (whooping cough), *Actinomyces israelii* (actinomycosis), *Streptococcus*

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*mutans* (dental caries), *Streptococcus equi* (strangles in horses), *Streptococcus agalactiae* (bovine mastitis), *Streptococcus anginosus* (canine genital infections).

(5) Viruses: Human immunodeficiency virus (HIV), poliovirus, influenza virus, rabies virus, herpes virus, foot and mouth disease virus, psittacosis virus, paramyxovirus, myxovirus, coronavirus.

Typically, the resulting immunological response occurs by both humoral and cell mediated pathways. One possible immunological response is the production of antibodies, thereby effecting protection against infection by the pathogen.

This method is not limited to protein antigens.

As discussed below, non-protein antigens or haptens can be covalently linked to the C-terminal cell-wall targeting segment, which can be produced as an independently expressed polypeptide, either alone, or with a spacer at its amino-terminal end. If a spacer at the amino-terminal end is used, typically the spacer will have a conformation allowing the efficient interaction of the non-protein antigen or hapten with the immune system, most typically a random coil or  $\alpha$ -helical form. The spacer can be of any suitable length; typically, it is in the range of about 5 to about 30 amino acids; most typically, about 10 to about 20 amino acids. In this version of the embodiment, the independently expressed polypeptide, once expressed, can then be covalently linked to the hapten or non-protein antigen. Typical non-protein antigens or haptens include drugs, including both drugs of abuse and therapeutic drugs, alkaloids, steroids, carbohydrates, aromatic compounds, including

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many pollutants, and other compounds that can be covalently linked to protein and against which an immune response can be raised.

Alternatively, a protein antigen can be covalently linked to the independently expressed cell-wall targeting segment or a cell-wall targeting segment including a spacer.

Many methods for covalent linkage of both protein and non-protein compounds to proteins are well known in the art and are described, for example, in P. Tijssen, "Practice and Theory of Enzyme Immunoassays" (Elsevier, Amsterdam, 1985), pp. 221-295, and in S.S. Wong, "Chemistry of Protein Conjugation and Cross-Linking" (CRC Press, Inc., Boca Raton, FL 1993).

Many reactive groups on both protein and non-protein compounds are available for conjugation.

For example, organic moieties containing carboxyl groups or that can be carboxylated can be conjugated to proteins via the mixed anhydride method, the carbodiimide method, using dicyclohexylcarbodiimide, and the N-hydroxysuccinimide ester method.

If the organic moiety contains amino groups or reducible nitro groups or can be substituted with such groups, conjugation can be achieved by one of several techniques. Aromatic amines can be converted to diazonium salts by the slow addition of nitrous acid and then reacted with proteins at a pH of about 9. If the organic moiety contains aliphatic amines, such groups can be conjugated to proteins by various methods, including carbodiimide, tolylene-2,4-diisocyanate, or maleimide compounds, particularly the N-hydroxysuccinimide esters



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of maleimide derivatives. An example of such a compound is 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid. Another example is m-maleimidobenzoyl-N-hydroxysuccinimide ester. Still another reagent that can be used is N-succinimidyl-3-(2-pyridyldithio) propionate.

Also, bifunctional esters, such as dimethylpimelimidate, dimethyladipimidate, or dimethylsuberimidate, can be used to couple amino-group-containing moieties to proteins.

Additionally, aliphatic amines can also be converted to aromatic amines by reaction with p-nitrobenzoylchloride and subsequent reduction to a p-aminobenzoylamide, which can then be coupled to proteins after diazotization.

Organic moieties containing hydroxyl groups can be cross-linked by a number of indirect procedures. For example, the conversion of an alcohol moiety to the half ester of succinic acid (hemisuccinate) introduces a carboxyl group available for conjugation. The bifunctional reagent sebacoyldichloride converts alcohol to acid chloride which, at pH 8.5, reacts readily with proteins. Hydroxyl-containing organic moieties can also be conjugated through the highly reactive chlorocarbonates, prepared with an equal molar amount of phosgene.

For organic moieties containing ketones or aldehydes, such carbonyl-containing groups can be derivatized into carboxyl groups through the formation of O-(carboxymethyl) oximes. Ketone groups can also be derivatized with p-hydrazinobenzoic acid to produce carboxyl groups that can be conjugated to the specific binding partner as described above. Organic moieties

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containing aldehyde groups can be directly conjugated through the formation of Schiff bases which are then stabilized by a reduction with sodium borohydride.

One particularly useful cross-linking agent for  
5 hydroxyl-containing organic moieties is a photosensitive noncleavable heterobifunctional cross-linking reagent, sulfosuccinimidyl 6-[4'-azido-2'-nitrophenylamino] hexanoate. Other similar reagents are described in S.S. Wong, "Chemistry of Protein Conjugation and Cross-  
10 Linking," supra.

Other cross-linking reagents can be used that introduce spacers between the organic moiety and the specific binding partner.

These methods need not be described further  
15 here.

Another use of the chimeric proteins and complexes of the present invention is in the biotechnological field of bacterial cell surface display.

If these molecules are associated with cell surfaces,  
20 they can be used in various ways for screening. For example, samples of expressed proteins from an expression library containing the expressed proteins on the surfaces of the cells can be used to screen for clones that express a particular desired protein when a labeled  
25 antibody or other labeled specific binding partner for that protein is available.

In general, such a method comprises:

1) incorporating a cloned protein into a chimeric protein including therein a carboxyl-terminal  
30 cell wall-targeting signal;

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(2) binding the chimeric protein to the surface of a Gram-positive bacterium to form a stable noncovalent complex; and

(3) reacting the noncovalent complex with a  
5 labeled specific binding partner to screen the chimeric protein for reactivity with the specific binding partner.

As used herein, the term "incorporating" means to include in a single molecule, typically a fusion protein. The fusion protein is formed by methods well  
10 known in the art and can include a spacer.

As used herein, the term "antibody" includes both intact antibody molecules of the appropriate specificity and antibody fragments (including Fab, F(ab'), Fv, and F(ab')<sub>2</sub> fragments) as well as chemically modified  
15 intact antibody molecules and antibody fragments, including hybrid antibodies assembled by in vitro reassociation of subunits. Also included are single-chain antibody molecules generally denoted by the term sFv and humanized antibodies in which some or all of the  
20 originally non-human constant regions are replaced with constant regions originally derived from human antibody sequences. Both polyclonal and monoclonal antibodies are included unless otherwise specified.

In the last step, the cells are merely exposed  
25 to the labeled antibody or other labeled specific binding partner, unreacted antibody is removed as by a wash, and label associated with the cells is detected by conventional techniques such as fluorescence, chemiluminescence, or autoradiography. As described  
30 above, this is an improvement on phage display.

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Cell-wall targeted molecules can also be used for the diagnosis and treatment of bacterial infections caused by Gram-positive bacteria. Such infections continue to present a serious medical challenge, particularly because of the spread of antibiotic resistance strains of Gram-positive bacteria. Antibiotic molecules or fluorescent or any other diagnostic molecules can be chemically linked to a cell-wall targeting peptide segment, which may include a spacer as described above, and then can be injected into animals or humans. The cell-wall targeted molecules are specifically directed to the bacterial target, thereby avoiding all host cells and fulfilling the specificity requirements of a "magic bullet."

In general, these methods comprise:

- (1) conjugating an antibiotic or a detection reagent to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate;
- (2) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the conjugate noncovalently to the cell walls of the bacterium in order to treat or diagnose the infection.

The antibiotic used can be, but is not limited to, a penicillin, ampicillin, vancomycin, gentamicin, streptomycin, a cephalosporin, amikacin, kanamycin, neomycin, paromomycin, tobramycin, ciprofloxacin, clindamycin, rifampin, chloramphenicol, or norfloxacin.

The detection reagent is typically an antibody or other specific binding partner labeled with a detectable label, such as a radiolabel. Such methods are

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well known in the art and need not be described further here.

In this context, the conjugates can be administered using conventional modes of administration including, but not limited to, intravenous, intraperitoneal, oral, or intralymphatic. Other routes of injection can alternatively be used. Oral or intraperitoneal administration is generally preferred. The composition can be administered in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends on the mode of administration and a quantity administered.

The compositions for administration preferably also include conventional pharmaceutically acceptable carriers and adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate. The most effective mode of administration and dosage regimen for the conjugates as used in the methods of the present invention depend on the severity and course of the disease, the patient's health, the response to treatment, the particular strain of bacteria infecting the patient, other drugs being administered and the development of resistance to them, the accessibility of the site of infection to blood flow, pharmacokinetic considerations such as the condition of the patient's liver and/or kidneys that can affect the

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metabolism and/or excretion of the administered conjugates, and the judgment of the treating physician. Accordingly, the dosages should be titrated to the individual patient.

5

#### IV. NOVEL COMPOUNDS ACCORDING TO THE PRESENT INVENTION

Another aspect of the present invention is novel compounds embodying the concept of the present invention. These novel compounds include: (1) chimeric  
10 proteins including a protein to be targeted and a cell-wall targeting signal; (2) DNA or other nucleic acid segments encoding such chimeric proteins; (3) vectors incorporating the DNA or other nucleic acid segments encoding the chimeric proteins; (4) stable noncovalent  
15 complexes of bacterial cells and cell-wall targeted proteins; (5) covalent conjugates of protein or non-protein antigens and cell-wall targeted proteins, optionally including a spacer; and (6) noncovalent complexes of these conjugates and Gram-positive bacteria.

20 The production of these novel compounds according to the present invention is as described above.

In general, chimeric proteins according to the present invention comprise:

(1) a protein to be targeted to the cell wall  
25 of a Gram-positive bacterium; and

(2) a cell-wall targeting signal located at the carboxyl terminus of the chimeric protein for binding the chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting  
30 signal so that the chimeric protein is displayed on the

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surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

Nucleic acid segments encoding the chimeric proteins of the present invention can be either DNA or RNA; typically, they are DNA. The term "nucleic acid" as used herein includes both DNA and RNA as well as DNA-RNA hybrids unless otherwise indicated, and can include both single and double-stranded nucleic acid sequences. If a DNA sequence is referred to, reference is generally to both strands of a DNA sequence, either individually or as a Watson-Crick double helix. If only one strand is specified, the complementary strand whose antiparallel sequence is determined by Watson-Crick based pairing rules is also included unless the complementary sequence is specifically excluded. If only one strand is specified in double-stranded DNA, the strand specified is the sense strand whose strand would be equivalent to the sequence of any RNA transcribed from the double-stranded DNA, except for the replacement of thymidine (T) in the DNA by uridine (U) in the RNA. Reference to a nucleic acid sequence also includes modified bases as long as the modification does not significantly interfere with Watson-Crick base pairing or other specified functions of the nucleic acid, and can, for example, include substitution of uridine for thymidine in DNA as well as methylation of bases or modification of sugars.

Construction of nucleic acid segments according to the present invention can be accomplished by techniques well known in the art including solid-phase nucleotide synthesis, the polymerase chain reaction (PCR) technique, reverse transcription of DNA from RNA, the use of DNA

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polymerases and ligases, and other techniques. If an amino acid sequence is known, the corresponding nucleic acid segment can be constructed according to the genetic code.

5           Another aspect of the invention is a vector comprising the nucleic acid segment operatively linked to at least one control sequence that controls the expression or regulation of the nucleic acid segment. Such control sequences are well known in the art and include operators, 10 promoters, enhancers, promoter-proximal elements, and replication origins. The techniques of vector construction, including cloning, ligation, gap-filling, the use of the polymerase chain reaction (PCR) procedure, solid-state oligonucleotide synthesis, and other 15 techniques, are also well known in the art and need not be described further here.

          Another aspect of the present invention is noncovalent complexes comprising chimeric proteins according to the present invention and a Gram-positive 20 bacterium. In the complex, the chimeric protein is bound noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is 25 accessible to a ligand.

          Yet another aspect of the present invention is a covalent conjugate comprising:

          (1) a cell-wall targeting segment including a carboxyl-terminal cell-wall targeting signal therein that 30 can be bound noncovalently and stably to a cell wall of a



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Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal; and

(2) an antigen or hapten conjugated covalently to the cell-wall targeting segment.

5 In these conjugates, the antigen or hapten can be a protein antigen; alternatively, it can be a nonprotein antigen or hapten. The cell-wall targeting segment can further include an amino-terminal spacer.

10 Yet another aspect of the present invention is a noncovalent complex comprising the covalent conjugate and a Gram-positive bacterium stably and noncovalently binding the covalent conjugate.

15 The invention is further described by means of the following Example. This Example is for illustrative purposes only and is not to be construed as limiting the scope of the invention in any manner.

#### EXAMPLE

20 Targeting of Proteins to the Staphylococcal  
Cell Wall by Cell-Wall Targeting Segments Derived  
from Lysostaphin and Amidase

25 Bacteriocin or antibiotic molecules cause the selective killing of sensitive bacteria and are thought to provide an advantage for the growth and survival of the producing microorganism (R. Kolter, "Genetics of Ribosomally Synthesized Peptide Antibiotics," Annu. Rev. Microbiol. 46: 141-163 (1992). Investigations into the mode of action of bacteriocins led to the discovery and  
30 elucidation of fundamental biological processes such as DNA replication (M.C. Garrido et al., "The Export of the

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DNA Replication Inhibitor Microcin B17 Provides Immunity for the Host Cell," EMBO J. 7: 1853-1862 (1988)), membrane transport (F. Pattus et al., "Colicins: Prokaryotic Killer-Pores," Experientia 46: 180-192 (1990)), and cell wall biosynthesis (J.L. Strominger et al., "Peptidoglycan Transpeptidase and D-Alanine Carboxypeptidase: Penicillin-Sensitive Enzymatic Reactions," Fed. Proc. 26: 9-18 (1967)). These studies also provided tangible results in that they supplied therapeutics for the treatment of human infections caused by bacterial pathogens. One important question in understanding the mode of action of antibiotics is the mechanism of target cell specificity that allows the bacteriocin to distinguish between the host and the target cell. Some colicins, for example, kill sensitive *Escherichia coli* cells by forming voltage-dependent ion channels after their insertion into the cytoplasmic membrane (Pattus et al. (1990), supra). Colicin-sensitive cells harbor a specific receptor protein in the outer membrane that allows the binding and specific import of the bacteriocin across the outer envelope of these strains (H. Benedetti et al., "Individual Domains of Colicins Confer Specificity in Colicin Uptake, In Pore-Properties and in Immunity Requirement," J. Mol. Biol. 217: 429-439 (1991)). Colicin-producing *E. coli* cells require an immunity determinant for protection against the secreted bacteriocin (H. Pils1 & V. Braun, "Evidence That the Immunity Protein Inactivates Colicin 5 Immediately Prior to the Formation of the Transmembrane Channel," J. Bacteriol. 177: 6966-6972 (1995)).

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In contrast to the well-studied bacteriocins from Gram-negative bacteria, very little is known about the target cell specificity of antibiotic molecules directed against Gram-positive organisms. Most bacteriocins directed against Gram-positive cells are in fact small peptides, or microcins, that diffuse through the bacterial cell wall and exert their bactericidal action by inserting into the cytoplasmic membrane (R.W. Jack et al., "Bacteriocins of Gram-Positive Bacteria," Microbiol. Rev. 59: 171-200 (1995)). Because many of these molecules display broad killing activity for a wide variety of bacterial species, it is questionable whether or not these microcins display target cell specificity. Microcin-secreting organisms therefore require specific immunity proteins for protection from the bactericidal activity, but the molecular mechanisms of immunity have not been resolved yet (R.W. Jack et al. (1995), supra)).

The Gram-positive organism *Staphylococcus simulans* secretes lysostaphin, a bacteriolytic enzyme that cleaves the pentaglycine cross bridges of staphylococcal peptidoglycans (C.A. Schindler & V.T. Schuhardt (1964), supra). Pre-pro-lysostaphin is exported from the cytoplasm by an N-terminal signal peptide (P.A. Recsei et al. (1987), supra; P. Heinrich et al., "The Molecular Organization of the Lysostaphin Gene and Its Sequences Repeated in Tandem," Mol. Gen. Genet. 209:563-569 (1987)). Subsequently, 14 tandem repeats of a 13-residue peptide are cleaved from the N-terminus of the secreted pro-enzyme, thereby generating mature lysostaphin in the culture medium (P.A. Recsei et al. (1987), supra; P. Heinrich et al. (1987), supra). The pentaglycine

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crossbridges, the substrates for the enzymatic digestion by lysostaphin (H.P. Browder et al., "Lysostaphin: Enzymatic Mode of Action," Biochem. Biophys. Res. Commun. 19:383-389 (1965); G.L. Sloan et al., "Lysostaphin  
5 Endopeptidase-Catalyzed Transpeptidation Reactions of the Imino-Transfer Type," Biochem. J. 167:293-296 (1977)), are integral components of staphylococcal peptidoglycans and occur in both organisms, *S. simulans* and *S. aureus* (K.H. Schleifer & O. Kandler, "Peptidoglycan Types of  
10 Bacterial Cell Walls and Their Taxonomic Implications," Bacteriol. Rev. 36:407-477 (1972)), yet *S. simulans* host cells are not killed by the secreted bacteriocin.

It is demonstrated here that the information for target cell specificity is encoded within the  
15 lysostaphin molecule. A C-terminal sequence element of lysostaphin is both necessary and sufficient for targeting to the cell wall of *S. aureus*; this sequence element is the C-terminal cell-wall targeting signal described above.

20 Sequence elements homologous to the cell-wall targeting signal were found in several other cell-wall proteins of gram-positive bacteria (A. Wang et al. (1991), supra; V.C. Neuman et al., FEMS Microbiol. Lett. 110:205 (1993)). One of these elements, the targeting  
25 signal of staphylococcal amidase (LytA), also functions to direct chimeric fusion proteins to the cell wall of *S. aureus*. Deletion of the targeting signal did not interfere with endopeptidase activity but abolished the bacteriolytic killing of *S. aureus* cells, indicating that  
30 this domain functions to specifically address the bacteriocin molecule to its target cells.

## Results

To understand the mechanism for both targeting of lysostaphin to the cell wall of *S. aureus* and the immunity of *S. simulans* cells to secreted lysostaphin, the export, processing, and cell location of lysostaphin expressed in both of these organisms was analyzed.

To prevent the bacteriolytic killing of *S. aureus*, the lysostaphin gene (*lst*) was cloned under control of the *BlaZRI* regulon (P.-Z. Wang et al., Nucl. Acids Res. 19:4000 (1991)) and expression was induced by the addition of the  $\beta$ -lactam antibiotic methicillin to staphylococcal cultures. The plasmid pLST was assembled from three components: (1) the lysostaphin gene; (2) *blaZRI* sequences encoding the repressor (*blaI*), the signal transducer (*blaR*), as well as the promoter of the  $\beta$ -lactamase regulon; and (3) the *E. coli-S. aureus* shuttle vector pOS1 (O. Schneewind et al., "Cell Wall Sorting Signals in Surface Protein of Gram-Negative Bacteria," EMBO J. 12: 4803-4811 (1993)). The *lst* gene was amplified by the polymerase chain reaction (PCR) using *S. simulans* ATCC1362 DNA as a template with the primers LS-Nde (5'-AACATATGAAGAAAACAAAAACAATTATTATA-3') (SEQ ID NO: 5) and LS-Bam (5'-AAGGATCCTCACTTTATAGTTCCCCAAA-3') (SEQ ID NO: 6) and digested with *NdeI* and *BamHI*. The *blaZRI* sequences were amplified from pI258 (R.P. Novick & M.H. Richmond, "Nature and Interactions of the Genetic Elements Governing Penicillinase Synthesis in *Staphylococcus aureus*," J. Bacteriol. 90: 467-480 (1965)) with the

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primers Bla-Pro-Nde (5'-TCCCCCGGGCATATGAAACCCTCCGATATTACAGTT-3') (SEQ ID NO: 7) and Bla-18 (5'-AAGAATTCCATATCTCTAATTCATTTTTACTAA-3') (SEQ ID NO: 8) and digested with *Nde*I and *Eco*RI. Finally, 5 pOS1 was digested with *Eco*RI and *Bam*HI and all three fragments were ligated and electroporated into *S. aureus* OS2. The *Nde*I (CATATG) and *Bam*HI sites of pLST allowed the precise insertion of the *lst* gene at the start codon and stop codons of *bla*Z.

10 Staphylococcal cultures were pulse-labeled with [<sup>35</sup>S]methionine and chased by adding non-radioactive methionine. During the pulse or at timed intervals of the chase, aliquots of the culture were precipitated with ice-cold trichloroacetic acid (TCA). The staphylococcal 15 peptidoglycan was digested with *Chalaropsis* muramidase (J.H. Hash, "Purification and Properties of Staphylolytic Enzymes from *Chalaropsis* sp.," Arch. Biochem. Biophys. 102: 379-388 (1963)) and the samples were re-precipitated with TCA followed by immunoprecipitation with anti- 20 lysostaphin prior to SDS polyacrylamide gel electrophoresis.

The following procedures were used in the experiments whose results are shown in Figures 1-3. Staphylococcal cultures were grown overnight in 25 chemically defined medium (L. van de Rijn & R.E. Kessler, "Growth Characteristics of Group A Streptococci in a New Chemically Defined Medium," Infect. Immun. 27: 444-448 (1980)), diluted 1:20 into minimal medium. The cultures were pulse-labeled when they reached OD<sub>600</sub> 0.5. The 30 expression of lysostaphin in *S. aureus* was induced with 1

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$\mu$  methicillin 15 min prior to pulse-labeling. After labeling with 100  $\mu$ Ci [ $^{35}$ S] methionine for 2 min, the pulse was chased with 50  $\mu$ l chase solution (100 mg casamino acids, 10 mg methionine/ml) for up to 60 min.

5 For pulse chase experiments, at timed intervals during either the pulse or the chase, 200  $\mu$ l aliquots of the culture were precipitated with 200  $\mu$ l ice-cold 10% TCA. The TCA precipitate was washed in acetone, dried, digested with muramidase for 2 h at 37°C (1 ml 0.05 M

10 sodium acetate, pH 5.7, 100  $\mu$ l muramidase) and the samples were again precipitated with TCA. For analysis of secreted and cell associated lysostaphin, cells were recovered by centrifugation for 4 min at 15,000 x g and the supernatant was removed and precipitated with TCA

15 (secreted fraction). The cell pellet was digested with muramidase for 10 min at 37°C in 1 ml STM buffer (0.5 M sucrose, 0.02 M Tris-HCl, 0.02 MgCl<sub>2</sub>, pH 7.5, 100  $\mu$ g muramidase) and precipitated by the addition of TCA (cell associated fraction). For cell fractionations, 1 ml of

20 staphylococcal culture expressing SEB fusions was pulse labeled and the cells were recovered by centrifugation for 4 min at 15,000 x g. The supernatant was removed and precipitated with TCA (medium) and the cells were

lysostaphin digested for 10 min at 37°C in 500  $\mu$ l SMM

25 buffer (0.5 M sucrose, 0.02 M maleate, 0.02 M MgCl<sub>2</sub>, pH 6.5, 100  $\mu$ g lysostaphin). The protoplasts were collected by centrifugation for 4 min at 15,000 x g and the supernatant was removed and precipitated with TCA (cell

30 wall fraction). The protoplasts were lysed in 250  $\mu$ l membrane buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.01 M MgCl<sub>2</sub>,

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pH 7.5) with five cycles of freeze-thawing in a dry ice-ethanol bath. The membranes were pelleted by ultracentrifugation in a Beckman TL-100 ultracentrifuge at 100,000 x g for 30 min. The supernatant (cytoplasm) and the pellet (membranes) were separated and precipitated with TCA. To determine solubility in hot SDS, 1 ml of culture was pulse-labeled and chased for 5 min, split into two aliquots of 500  $\mu$ l each, and precipitated with TCA. After TCA precipitation and an acetone wash, one of the two chase samples was lysostaphin digested (500  $\mu$ l 0.5 M Tris-HCl, 100  $\mu$ g lysostaphin) for 30 min at 37°C and again precipitated with TCA prior to boiling in hot SDS. The other of the two aliquots was directly boiled in hot SDS. All TCA precipitated samples were washed in acetone, dried, and boiled in 50  $\mu$ l 0.5 M Tris-HCl, pH 8.0, 4% SDS. Soluble material was immunoprecipitated prior to SDS-PAGE. For immunofluorescence, 1 ml of midlog staphylococcal culture ( $OD_{600}$  0.5) grown in tryptic soy broth was centrifuged (5 min at 15,000 x g) and the cells were washed twice in ice-cold water and finally suspended in 1 ml phosphate buffered saline (PBS, 0.02% sodium azide). The cell suspension (5  $\mu$ l) was applied to a glass slide, air dried and heat fixated. The cells were incubated with 50  $\mu$ l of FITC-labeled rabbit immunoglobulin (Sigma, St. Louis, MO) diluted 1:20 in PBS. The glass slides were washed several times in PBS and finally evaluated by photomicrography under ultraviolet light at 1000-fold magnification.



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In its natural host, *S. simulans*, pulse-labeled pre-pro-lysostaphin was rapidly secreted into the culture medium; the enzyme was exported and cleaved to the pro-form within 2 min of its synthesis. The processing of pro-lysostaphin to the mature enzyme occurred much more slowly, requiring more than 60 min (Fig. 1); the half-time for processing was 60 min. *S. simulans* culture supernatants were analyzed for the presence of pro- and mature lysostaphin during logarithmic growth and stationary phase. It was found that the processing of pro-lysostaphin occurred almost exclusively during stationary phase. When expressed in *S. aureus*, pre-pro-lysostaphin was converted very rapidly to the mature species ( $t_{1/2} < 30$  sec) without a detectable pro-lysostaphin intermediate. Pulse-labeled lysostaphin could only be immunoprecipitated within 5 min of the pulse, indicating that when synthesized by *S. aureus* cells the bacteriocin was rapidly degraded. The addition of *S. simulans* culture medium containing pulse-labeled pro-lysostaphin to *S. aureus* cells did not increase the processing of the pro-form to the mature species.

To distinguish secreted from cell-associated lysostaphin, staphylococci were centrifuged and the medium (MD) was separated from the cells ( $\Phi$ ). The cell pellet was subjected to peptidoglycan degradation with muramidase, and both medium and pellet fractions were precipitated with TCA. In pulse-labeled *S. simulans* cultures, lysostaphin was found exclusively in the culture medium, whereas all lysostaphin immunoprecipitated from *S. aureus* was located in the cell pellet fraction (Figure 1). TCA precipitation of

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staphylococcal cultures followed by suspension in hot SDS causes the solubilization of only those proteins that are secreted into the medium, whereas all cellular proteins remain insoluble unless the thick peptidoglycan layer of these organisms has been degraded (O. Schneewind et al., "Sorting of Protein A to the Staphylococcal Cell Wall," Cell 70: 267-281 (1992)). This property was exploited and the secretion of lysostaphin was measured by comparing its solubility in hot SDS either with (H) or without prior peptidoglycan degradation (CH). Lysostaphin secreted into the medium of *S. simulans* cultures was directly soluble in hot SDS. In contrast, all lysostaphin synthesized in *S. aureus* cells required prior peptidoglycan degradation for solubility in hot SDS, indicating that lysostaphin remained cell-associated and trapped within the staphylococcal cell wall.

In Fig. 1, panel (A), the drawing shows the structures of wild-type lysostaphin (LST) and its mutants. Pre-pro-lysostaphin consists of a N-terminal leader peptide (open bar) that is thought to be responsible for protein export from the cytoplasm. Fourteen N-terminal tandem repeats (NTR) of a 13-residue peptide (NH<sub>2</sub>-AEVETSAPVENT-COOH) (SEQ ID NO: 9) are cleaved from pro-lysostaphin to generate the mature enzyme, which consists of an enzyme domain (central open bar) and a C-terminal cell-wall targeting signal (hatched bar, CWT). To identify a specific cell-wall targeting signal, mutant lysostaphin molecules with a deletion of either the C-terminal domain (LST<sub>ΔCWT</sub>) or the 14 N-terminal repeats (LST<sub>ΔNTR</sub>) were constructed.

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In panel (B), pulse-chase experiments are shown. During pulse-chase experiments, staphylococci were pulse-labeled with [<sup>35</sup>S] methionine for 2 min followed a chase of non-radioactive methionine. Aliquots of the pulse-labeled culture were precipitated with trichloroacetic acid (TCA) during the pulse (0) and 1, 5, 20, or 60 min after the addition of the chase. Pulse-labeled lysostaphin was immunoprecipitated with a specific antiserum, separated on 12% SDS-polyacrylamide gel electrophoresis and fluorographed. In *S. aureus* cells, expression of LST and its truncated forms, genes which were under the control of the BlaZRI regulon, were induced by 1  $\mu$ M of methicillin 15 min prior to pulse labeling (right panel). In cell-fractionation experiments, pulse-labeled cultures were fractionated into medium (MD) and whole cell pellet ( $\Phi$ ) and precipitated with TCA. Proteins in the cell pellet ( $\Phi$ ) were released from the peptidoglycan by treatment with 100  $\mu$ g/ml of Chalaropsis B muramidase (Hash-enzyme) and again precipitated with TCA. In parallel, pulse-labeled cultures were precipitated with TCA in duplicate. To solubilize proteins secreted into the staphylococcal medium, one of the samples was directly boiled in hot SDS (CH) while the other sample was digested with hash-muramidase prior to TCA precipitation (H), a protocol that solubilizes the cellular proteins of staphylococci.

Figure 1(C) shows the results of an experiment indicating that secreted LST from *S. simulans* cells was not cleaved rapidly even in the presence of *S. aureus* cells. A mid-log culture of *S. simulans* was pulse-

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labeled for 2 min and the culture supernatant containing secreted LST was immediately removed after collecting the cells by centrifugation. The supernatant was then added to the uniradiolabeled cell pellet of *S. aureus* (left) or  
5 *S. simulans* (right) which were separated from mid-log cultures. After suspending the cells in the radiolabeled LST-containing supernatant, samples were incubated for indicated periods (in min) at 37°C. At each period, reaction was stopped by adding TCA. Precipitated  
10 materials were solubilized in hot SDS and then subjected to immunoprecipitation in SDS-polyacrylamide gel electrophoresis as in (B).

The C-terminal 92 residues of lysostaphin display sequence homology to the C-terminal domains of  
15 staphylococcal amidase (LytA) and cell-wall proteins of *Staphylococcus mutans* (A. Wang et al. (1991), supra). A mutant lysostaphin was therefore constructed with a C-terminal truncation of 92 residues (LST<sub>ΔCWT</sub>). It was  
20 surprising that this mutant lysostaphin was secreted into the culture medium of *S. aureus*. Cleavage of the mutant precursor to the pro-form was slower ( $t_{1/2}$  = 2 min) and processing of its fourteen tandem repeats required more than 60 min, rates similar to those measured for wild-type lysostaphin secreted into the culture medium of *S.*  
25 *simulans*. These results indicated that the C-terminal 92 residues of lysostaphin were required for bacteriocin targeting to the envelope of *S. aureus* cells as well as for the rapid processing of the pro-form.

The 14 N-terminal repeats of pro-lysostaphin  
30 were not required for cell-wall targeting, because a

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mutant lacking the sequences (LST<sub>ANTR</sub>) was located in the cell-wall compartment of *S. aureus* similar to the targeting of wild-type lysostaphin. Therefore, deletion of the 14 N-terminal repeats did not alter the cellular location of the mutant molecule.

To see whether the C-terminal domain of lysostaphin was sufficient for the targeting of indicator molecules to *S. aureus* cells, enterotoxin B (SEB) was employed. This is a protein normally secreted into the staphylococcal medium (R.K. Tweten & J.J. Iandolo, "Transport and Processing of Staphylococcal Enterotoxin B," J. Bacteriol. 153: 297-303 (1983)) (Figure 2). The cellular location of hybrid SEB molecules was analyzed by fractionating pulse-labeled staphylococcal cultures into the medium, cell wall, cytoplasm, and membrane components. A chimeric protein with a fusion of the C-terminal domain of lysostaphin to the C-terminus of enterotoxin B (SEB-LST) was located in the cell wall compartment of *S. aureus* and was only soluble in hot SDS after the peptidoglycan had been degraded (Figure 2). As a control for the correct fractionation of staphylococci, the studies included indicator molecules that were either secreted into the medium (SEB), covalently linked to the peptidoglycan (SEB-SPA<sub>490-524</sub>) or membrane anchored (SEB-ACTA) (O. Schneewind et al., "Cell Wall Sorting Signals in Surface Protein of Gram-Positive Bacteria," EMBO J. 12: 4803-4811 (1993)).

Surface proteins of *S. aureus* are covalently linked to the bacterial peptidoglycan via a C-terminal sorting signal. The covalent linkage of proteins to the cell wall can be measured by specifically cleaving the

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staphylococcal peptidoglycan with two different enzymes (J.-M. Ghuysen, "Use of Bacteriolytic Enzymes in Determination of Wall Structure and Their Role in Cell Metabolism," Bacteriol. Rev. 32: 425-464 (1968)), thereby  
5 releasing anchored protein with different amounts of linked cell wall (Schneewind et al. (1993), supra). To test for a peptidoglycan linkage of SEB-LST, the cell  
wall of pulse-labeled was digested with either lysostaphin (L) or muramidase (H, Hash-enzyme) and  
10 compared the mass of immunoprecipitated proteins on SDS-PAGE (Fig. 2C). Peptidoglycan digestion with either lysostaphin or muramidase did not alter the migration of SEB-LST on SDS-PAGE, indicating that the hybrid molecule was not covalently linked to the staphylococcal cell  
15 wall. As a control for a cell wall linked protein, cleavage of the peptidoglycan with muramidase released SEB-SPA<sub>490-524</sub> as a spectrum of fragments with increasing mass, all of which migrated more slowly on SDS-PAGE than the lysostaphin-released counterpart (Schneewind et al.  
20 (1993), supra).

Taken together with the results above, this demonstrates that the C-terminal 92 residues of lysostaphin were necessary and sufficient for protein targeting to the cell wall of *S. aureus*. This element  
25 was therefore named the cell wall targeting (CWT) signal.

Previous work reported homology between the C-terminal sequences of staphylococcal amidase and lysostaphin (56% sequence identity) (A. Wang et al., "Sequence Analysis of a *Staphylococcus aureus* Gene Encoding a Peptidoglycan  
30 Hydrolase Activity," Gene 102: 105-109 (1991)). To ask whether the C-terminal domain of amidase (LytA) has a

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function similar to that of the lysostaphin targeting signal, the C-terminal amino acid region of LytA was fused to SEB; it was found that this hybrid molecule was also targeted to the cell wall of *S. aureus* (Fig. 2).

5 In Figure 2(A), the drawing shows the structures of enterotoxin B (SEB, 1) and its hybrid proteins with a C-terminal fusion of: (2) the cell-wall sorting signal of protein A which consists of the LPXTG (SEQ ID NO: 10) motif (LPETG) (SEQ ID NO: 11), the C-  
10 terminal hydrophobic domain (black bar) and the charged tail (+); (3) the membrane anchor segment of *Listeria monocytogenes* ActA (stippled bar); (4) the cell-wall targeting domain of lysostaphin (hatched bar) and (5) the cell-wall targeting domain (C-terminal 99 amino  
15 acids) of Staphylococcal amidase (LytA) (hatched bar).

In Figure 2(B), *S. aureus* OS2 expressing genes of various hybrid proteins was pulse-labeled with [<sup>35</sup>S] methionine for 1 min and chased with non-radioactive amino acids for 5 min. The culture was fractionated into  
20 medium (MD), cell wall (CW), cytoplasm (C) and membrane (M) compartments. In parallel, 1 ml of culture was pulse-labeled and chased, and 500 µl each precipitated with TCA. One of these samples was lysostaphin digested (L) prior to boiling in hot SDS, whereas the other  
25 sample, (CH), was directly boiled in hot SDS. Proteins were solubilized in hot SDS, immunoprecipitated with anti-SEB antibody, and subjected to 12% SDS-polyacrylamide gel electrophoresis.

In Figure 2(C), 1 ml of *S. aureus* OS2 culture  
30 expressing genes of SEB proteins was pulse-labeled with [<sup>35</sup>S] methionine for 1 min, chased with non-radioactive

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amino acids, and 500  $\mu$ l each precipitated with TCA. The two samples were digested with either lysostaphin (L), or Chalaropsis B muramidase (Hash-enzyme) (H), and precipitated with TCA. Proteins were solubilized in hot SDS, immunoprecipitated with anti-SEB, and subjected to 12% SDS-polyacrylamide gel electrophoresis. Molecular weight markers are indicated (kDa).

Upon cell fractionation, the hybrid SEB-LST was found mostly in the cell-wall compartment of *S. aureus*. This hybrid molecule was soluble in SDS and did not meet other criteria established for the covalent linkage of surface proteins for the peptidoglycan (O. Schneewind et al., (1993), supra), indicating that it was bound but not covalently linked to the staphylococcal cell wall. As a control for the correct fractionation of staphylococcal cells, hybrid SEB molecules known to be either covalently linked to the cell wall (SEB-SPA<sub>490-524</sub>) or membrane anchored (SEB-ActA) were included (O. Schneewind et al. (1993), supra).

The database was searched for sequence elements homologous to the cell-wall targeting signal of lysostaphin, and five cell-wall proteins harboring similar sequences were found. To analyze the function of one of these sequence elements, the cell-wall targeting signal of LytA was fused to the C-terminal end of SEB; it was found that this hybrid molecule was also targeted to the cell wall of *S. aureus*.

The hybrid molecules were bound but not covalently linked to the cell wall because the SEB-LST and SEB-LytA proteins do not display a size difference when solubilized from the staphylococcal cell wall with



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two different bacteriolytic enzymes (lysostaphin and Hash-enzyme). Because these enzymes cleave the cell wall at different points, if the chimeric proteins were covalently bound to the cell wall, they would be expected to display different molecular weights after cleavage because different portions of the cell wall would remain covalently attached. However, if the molecules are released from the cell wall by the digestion conditions without the necessity of cleaving any covalent bonds, the molecular weight would be expected to be identical. See panel (C), number 2 of Figure 2 as a control for a covalently linked molecule targeted by the protein A segment, which is known to result in covalent binding to the peptidoglycan layer of the cell wall (O. Schneewind et al., "Sorting of Protein A to the Staphylococcal Cell Wall," Cell 70:267-281 (1992)).

To investigate the cell surface display of molecules targeted to the cell wall of *S. aureus*, staphylococcal protein A was employed as an indicator molecule. Wild-type protein A is covalently linked to the staphylococcal cell wall by a mechanism requiring a C-terminal sorting signal (O. Schneewind et al. (1993), supra; O. Schneewind et al. (1992), supra). During cell-wall anchoring, the sorting signal is cleaved between the threonine (T) and the glycine (G) of an LPXTG (SEQ ID NO: 10) sequence motif (W.W. Navarre & O. Schneewind, Mol. Microbiol. 14:115 (1994)). The carboxyl of threonine is subsequently amide linked to the peptidoglycan crossbridge thereby anchoring the C-terminal end of the polypeptide chain to the peptidoglycan while the N-terminal immunoglobulin binding domains are displayed on

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the staphylococcal cell surface (O. Schneewind et al., 268:103 (1995)).

This comparison is shown in Figure 3, comparing the surface display of proteins targeted to the cell wall of *S. aureus* by covalent or noncovalent mechanisms. Specifically, to investigate if proteins targeted to the wall of *S. aureus* were displayed on the cell surface, the binding of FITC-labeled immunoglobulin to protein A located on the staphylococcal surface via UV light microscopy. The C-terminal end of wild-type protein A (SPA) is linked to and buried within the bacterial cell wall, whereas its N-terminal immunoglobulin binding domains bind FITC-labeled IgG on the cell surface as indicated by a strong fluorescent halo surrounding staphylococci (Fig. 3). Staphylococci expressing wild-type or mutant protein A were harvested by centrifugation, washed and incubated with fluorescein isothiocyanate labeled rabbit immunoglobulin (FITC-IgG). Binding of FITC protein A located on the staphylococcal surface was visualized by microscopy under ultraviolet light (1000x).

Figure 3 shows the results with *S. aureus* OS2 expressing either wild-type protein A (1), protein A harboring a C-terminal cell-wall targeting signal (SPA<sub>ΔCWT</sub>) (2) or a mutant protein A (SPA<sub>1-519</sub>) known to be secreted into the culture medium (3). The presence of staphylococci in panel (3) was demonstrated by light microscopy (3 visible light).

Wild-type protein A (SPA) bound FITC-labeled IgG on the staphylococcal surface as indicated by a strong fluorescent halo surrounding the staphylococci.

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When the C-terminal sorting signal of protein A, which generates covalent binding, was replaced with the cell-wall targeting signal of lysostaphin (SPA<sub>CWT</sub>), it was observed that the binding of FITC-labeled IgG to the staphylococcal surface occurred with intensity similar to that of wild-type protein A. This shows not only that the noncovalent binding induced by the cell-wall targeting signal of lysostaphin causes the targeted protein present in the chimeric protein to be displayed to the medium to allow the binding of specific binding partners such as antibodies, but that the strength of the noncovalent binding of the chimeric protein to the staphylococcal surface is sufficiently great to allow labeling comparable to that observed with a covalently bound protein A molecule.

As a negative control, no binding of FITC-labeled IgG to surface displayed protein A was observed for truncated protein A molecule (SPA<sub>1-519</sub>) that was secreted into the Staphylococcal culture medium (O. Schneewind et al. (1992), supra).

These results suggest that lysostaphin is specifically bound to the cell wall of *Staphylococcus aureus* by a C-terminal cell-wall targeting domain. This binding is noncovalent, but is sufficiently tight to allow use of the targeted protein for labeling. The targeted protein is accessible to the medium surrounding the cells for reaction with ligands, including specific binding partners such as antibodies.

Similar cell-wall targeting domains were found in several other cell-wall proteins, suggesting that

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these molecules may be targeted to the cell wall by a mechanism similar to that of lysostaphin.

A comparison of the sequences for LST and LytA, showing substantial homology between the C-terminal sequences of lysostaphin (LST) and amidase (LytA), is shown in Figure 4.

If the targeting signal of lysostaphin were sufficient to direct bacteriocin molecules to *S. aureus* cells, the addition of exogenous hybrid proteins to staphylococcal cultures should allow their targeting to *S. aureus* but not to *S. simulans* cells. This prediction was tested by purifying a hybrid glutathione S-transferase (D.B. Smith & K.S. Johnson, "Single-Step Purification of Polypeptides Expressed in *Escherichia coli* as Fusions with Glutathione S-Transferase," *Gene* 67: 31-40 (1988)) with a fused targeting signal (GST-CWT) and measuring its binding to *S. aureus* and *S. simulans* cells (Fig. 5). The binding of GST-CWT to staphylococci was quantitated by collecting cells with bound protein via centrifugation and measuring the decrease of glutathione S-transferase activity in the supernatant. *S. aureus* precipitated GST-CWT from the supernatant while *S. simulans* did not. Increasing amounts of *S. aureus* cells precipitated GST-CWT from the supernatant whereas *S. simulans* did not. Increasing amounts of *S. aureus* cells depleted increasing amounts of GST-CWT from the supernatant in a near linear manner ( $2 \times 10^7$  up to  $1 \times 10^8$  cfu). Nevertheless, increasing the number of *S. aureus* cells to more than  $1 \times 10^8$  cfu did not result in a further depletion of GST-CWT from the supernatant. This effect may be caused either by a population of GST-CWT molecules

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that is unable to bind *S. aureus* cells or by a receptor molecule that is partially soluble and hence does not allow the complete precipitation of bound GST-CWT.

In another assay, staphylococci were incubated  
5 with GST-CWT, collected by centrifugation, boiled in SDS, and soluble components were finally analyzed by SDS-PAGE.

Since staphylococci do not lyse in hot SDS this treatment results in the selective solubilization of GST-CWT bound to the cell surface. Unbound GST-CWT left in  
10 the supernatant was precipitated with TCA, separated on SDS-PAGE and all hybrid protein was identified by Coomassie staining. Increasing the concentration of *S. aureus* cells caused greater depletion of GST-CWT from the supernatant and accumulation in the cell pellet similar  
15 to that observed by measuring GST activity in cell supernatants. By dividing the molar amount of added fusion protein by the number of cells required for 50% precipitation, the number of binding sites for the targeting signal was calculated to be  $10^6$  per colony  
20 forming unit. No significant precipitation of GST-CWT fusion protein occurred when it was added to *S. simulans* cells.

For these experiments, *E. coli* strain XL-1 Blue harboring pGST-CWT was grown to mid-log phase in 500 ml  
25 of LB medium at 37°C, induced with 1 mM IPTG and incubated for another hour. Cells were harvested by centrifugation, suspended in 8 ml of F buffer (20% sucrose, 50 mM of Tris-HCl, pH 8.0, 1 mM DTT, 10 mM EDTA and 0.1 mg/ml of lysozyme) and incubated for 30 min at  
30 room temperature. The cells were disrupted by ultrasonication (W-200F instruments, Heat Systems-

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Ultrasonic Inc.). Unbroken cells were removed by centrifugation for 15 min at 15,000 x g and the supernatant was subjected to affinity chromatography on glutathione Sepharose 4G (Pharmacia), with a 1-ml column volume pre-equilibrated with PBS. The column was washed with 20 ml of PBS and eluted with 2 ml of 10 mM glutathione, 50 mM Tris-HCl, pH 8.0, yielding 200 µg/ml protein (approximate purity 98%). *S. aureus* OS2 or *S. simulans* ATCC1362 cells were grown to midlog phase (OD<sub>600</sub> 0.5) and washed several times with 50 mM Tris-HCl, pH 8.0. Purified GST-CWT protein (20 µg) was added to various amounts of staphylococci and incubated for 20 min at room temperature. The cells were collected by centrifugation (5 min at 15,000 x g) and the supernatant was removed from the pellet. The GST-CWT protein present in the supernatant was precipitated with 5% TCA, washed in acetone, and solubilized in 20 µl of sample buffer. The GST-CWT protein bound to staphylococci was eluted by adding 20 µl of sample buffer. All samples were boiled for 5 min and analyzed on 12% SDS-PAGE.

For the results shown in Figure 5, the hybrid protein with a fusion of the lysostaphin targeting signal to the C-terminus of GST-CWT was purified and 40 µg protein was added to indicated amounts of either *S. aureus* or *S. simulans* cells. GST-CWT protein bound to staphylococci was collected by centrifugation and separated from unbound protein located in the supernatant. In panel (A), the glutathione S-transferase activity in the supernatant was determined and plotted against the number of *S. aureus* (circles) and *S. simulans*

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(squares) cells. In panel (B), staphylococci with bound fusion protein were boiled in hot SDS and solubilized protein was precipitated on SDS-PAGE. The supernatant containing unbound GST-CWT protein was precipitated with:  
5 TCA and subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining.

Additionally, the targeting signal confers killing specificity onto lysostaphin. An assay was developed to specifically measure the bacteriolytic  
10 activity and target cell specificity of lysostaphin (Figure 6). Lysostaphin or its mutants were incubated with a mixture of *S. simulans* and *S. aureus* cells. and aliquots were plated on tryptic soy agar containing either erythromycin or streptomycin, thereby allowing the  
15 selective growth of either *S. aureus* (erythromycin resistant) or *S. simulans* (streptomycin resistant) survivors. For the experiments for which data is given in Figure 6, purified mature lysostaphin (LST), pro-lysostaphin (Pro-LST) or a mutant pro-lysostaphin lacking  
20 the C-terminal targeting signal (Pro-LST<sub>ΔCWT</sub>) were incubated with a suspension of *S. simulans* and *S. aureus* cells and the surviving bacteria were counted by plating on agar plates. The target specificity was calculated as the number of surviving host cells (*S. simulans*) divided  
25 by the number of surviving target cells (*S. aureus*). The amount of added enzyme (60 ng (LST) or 60 μg) (Pro-LST and Pro-LST<sub>ΔCWT</sub>) was adjusted to approximately  $3.5 \times 10^4$  units employing Acetyl-Gly<sub>6</sub> as a substrate. The results shown in Figure 6 are representative of three independent  
30 experiments.

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Purified mature lysostaphin ( $3.86 \times 10^4$  units) caused the selective killing of all but 4 out of  $10^5$  *S. aureus* target cells whereas only a few *S. simulans* cells were killed. The target cell specificity of mature  
5 lysostaphin was calculated as the number of surviving host cells (*S. simulans*) divided by the number of surviving target cells (*S. aureus*) and obtained a factor of  $5.3 \times 10^4$ .

To purify mutant bacteriocins, recombinant pro-  
10 and mature lysostaphin were expressed in *E. coli*. Measurements of lysostaphin activity in *E. coli* cell extracts revealed that recombinant pro-lysostaphin was active whereas recombinant mature lysostaphin was not, suggesting that the pro-region (14 N-terminal repeats)  
15 may function as an intramolecular chaperone for folding (X.L. Zhu et al., "Pro-Sequence of Subtilisin Can Guide the Refolding of Denatured Subtilisin in an Intermolecular Process," Nature 339: 483 (1989)). When  
20 tested with an acetylated hexaglycine residue (S.A. Kline et al., "A Colorimetric Microtiter Plate Assay for Lysostaphin Using a Hexaglycine Substrate," Anal. Biochem. 217: 329-331 (1994)), the purified pro-lysostaphin (Pro-LST) was significantly less active than  
25 mature lysostaphin isolated from *S. simulans*. This reduction is likely caused by the inefficient re-folding of pro-lysostaphin, because the recombinant molecule had been purified under denaturing conditions. Pro-LST ( $3.53 \times 10^4$  units) was tested for its bacteriolytic activity, and it was found that the targeting activity of the pro-  
30 enzyme was somewhat reduced (target cell specificity  $5.6 \times 10^2$ ). In contrast, a mutant pro-lysostaphin molecule



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lacking the C-terminal targeting signal (Pro-LST<sub>ΔCWT</sub>,  $3.23 \times 10^4$  units) had lost its ability to specifically destroy *S. aureus* cells (target cell specificity 1.3). This result indicated that the enzymatic endopeptidase activity of lysostaphin is not sufficient for the specific killing of *S. aureus* cells, but that the target specificity of this bacteriocin is dependent on its C-terminal targeting signal.

In these experiments, *E. coli* strain XL-1 Blue harboring either pPro-LST or pPro-LST<sub>ΔCWT</sub> was grown to midlog phase in 1 l of LB, induced with 1 mM IPTG, and incubated for another 3 hr. Cells were harvested by centrifugation, resuspended in buffer A (6 M guanidine-HCl, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0) and incubated for 1 hour at room temperature. After removal of cell debris by centrifugation for 15 min at  $10,000 \times g$ , the lysate was applied onto 1 ml Ni<sup>2+</sup>-NTA Sepharose (QIAGEN), pre-equilibrated with buffer A. The column was washed with 20 ml of buffer A, 20 ml of buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0), and 20 ml of buffer C (same composition as buffer B, but pH 6.3). Proteins were eluted with 5 ml of buffer E (same composition as buffer B, but pH 4.5). For renaturation and folding, the purified bacteriocins were dialyzed against 1 l of 0.05 M Tris-HCl, pH 7.5, 1 M urea, 0.005% of Tween 80 for 24 hours at 4°C without stirring, followed by another dialysis against the same buffer without urea for 16 hours at 4°C with slow stirring.

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Lysostaphin activity was measured as the release of free amino groups from N-acetylated hexaglycine (acetyl-Gly<sub>6</sub>) (S.A. Kline et al. (1994), supra). Briefly, 0.5 ml substrate solution (5 mM trisodium citrate, 1 mM disodium EDTA, 100 mM sodium borate, 10 mM acetyl-Gly<sub>6</sub>) and 0.5 ml of purified bacteriocin were mixed and incubated for 1 hour at 37°C. The colorimetric reagent 2,4,6-trinitrobenzenesulfonic acid (60 µl) in 0.1 M sodium bicarbonate was added and the reactions were incubated for 20 min at room temperature. The reactions were finally quenched with 100 µl of 3 M sodium acetate and absorbance at 405 nm was measured against a negative control with no added enzyme.

To determine the target cell specificity of lysostaphin, *S. aureus* OS2 (*spa*<sup>-</sup>, *ermC*) (O. Schneewind et al. (1992), supra), and *S. simulans* TNK1 (simultaneous streptomycin-resistant derivative of ATCC1362) were grown in TSB medium to mid-log phase. The cells were collected by centrifugation, washed and finally diluted to  $1.0 \times 10^6$  cfu/ml with fresh TSB. One ml of a 1:1 mixture of both diluted cultures was incubated with 0.5 ml of purified bacteriocin (60 ng of mature LST, 60 µg of Pro-LST, or 60 µg of Pro-LST<sub>ΔCWT</sub> in 50 mM Tris-HCl, pH 7.5, 0.005% of Tween 80) and incubated for 3 hours at 37°C. Aliquots of the reaction mixture were plated either directly or in a series of 10-fold dilutions on TSB agar containing the appropriate antibiotic (10 µg/ml of erythromycin or 250 µg/ml of streptomycin).

The targeting signal interferes with the sorting of surface proteins. Cell wall sorting signals,

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consisting of an LPXTG motif, hydrophobic domain and charged tail, cause the covalent anchoring of surface proteins to the Gram-positive cell wall (O. Schneewind et al. (1992), supra). During anchoring, the sorting signal is cleaved between the threonine and the glycine of the LPXTG (SEQ ID NO: 10) motif (W.W. Navarre & O. Schneewind, "Proteolytic Cleavage and Cell Wall Anchoring at the LPXTG Motif of Surface Proteins in Gram-Positive Bacteria," Mol. Microbiol. 14: 115-121 (1994)), and the liberated carboxyl of threonine is subsequently amide linked to a free amino in the pentaglycine crossbridge of the staphylococcal peptidoglycan (O. Schneewind et al., "Structure of the Cell Wall Anchor of Surface Proteins in *Staphylococcus aureus*," Science 268: 103-106 (1995)). An attempt was made to covalently link lysostaphin to its normal substrate, the pentaglycine crossbridge, by fusing the sorting signal of protein A to the C-terminal end of the polypeptide chain (LST-CWS (Figure 7)). The covalent linkage of the LST-CWS molecule was measured by digesting the peptidoglycan of *S. aureus* with muramidase. However, the hybrid LST-CWS molecule migrated uniformly on SDS-PAGE, suggesting that either the chimeric molecule was not linked to the peptidoglycan or that its own enzymatic activity caused its uniform solubilization from the peptidoglycan, a feature characteristically observed after lysostaphin digestion of cell wall linked proteins.

To distinguish between these two possibilities, the fact that the cleavage of the sorting signal at the LPXTG (SEQ ID NO: 10) motif causes cell wall-linked proteins to migrate faster on SDS-PAGE than their uncleaved and unanchored mutant counterparts was

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exploited. A hybrid lysostaphin molecule was constructed, with a fused sorting signal devoid of its LPETG (SEQ ID NO: 11) sequence (LST-CWS<sub>ΔLPETG</sub>); it was found that this molecule migrated faster on SDS-PAGE than the LST-CWS protein harboring a wild-type sorting signal.

This result indicated that the sorting signal of the LST-CWS protein had not been cleaved and that the molecule had therefore not been linked to the peptidoglycan of *S. aureus*.

To determine whether the sorting failure of LST-CWS was due to a general property of lysostaphin, the cell wall linkage of another protein harboring both targeting and sorting signals was tested (Figure 7). The sorting signal of protein A was fused to the C-terminus of SEB-LST. When solubilized with lysostaphin, two different SEB-LST-CWS species were observed on SDS-PAGE.

The slower migrating species was also present in muramidase-solubilized samples and it was therefore not anchored to the staphylococcal cell wall. In contrast, the faster migrating species of lysostaphin-digested samples was solubilized as a spectrum of fragments with increasing mass, indicating that part of SEB-LST-CWS protein had been linked to the peptidoglycan.

Furthermore, the unanchored species of SEB-LST-CWS protein migrated more slowly on SDS-PAGE than a control molecule with a defective LPXTG (SEQ ID NO: 10) motif, which suggests that its sorting signal had not been cleaved. Thus, the targeting signal of lysostaphin was at least in part responsible for the failure of the sorting signal to link lysostaphin to the staphylococcal cell wall.

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In the experiments shown in Figure 7, in panel (A) the drawing displays the structures of wild-type lysostaphin (LST, 1) and hybrid proteins with a C-terminal fusion of: (2) the cell wall sorting signal of protein A; (3) a sorting signal devoid of its LPXTG (SEQ ID NO: 10) motif. In addition, hybrid enterotoxin B molecules were constructed with a C-terminal fusion of both the lysostaphin targeting signal and the protein A sorting signal (4) or a similar hybrid molecule without an LPXTG (SEQ ID NO: 10) motif (5). When fused to exported proteins, the sorting signal causes cleavage at the LPXTG (SEQ ID NO: 10) motif and the linkage of the polypeptide chain to the bacterial peptidoglycan. In panel (B), the cell wall linkage of the hybrid proteins was analyzed by digesting pulse labeled staphylococci with muramidase (H), which solubilizes anchored proteins as a spectrum of fragments with increasing mass due to linked peptidoglycan fragments (see Fig. 2C, sample 2). As a control, TCA precipitated samples were either boiled in hot SDS (CH) or subjected to lysostaphin digestion (L). Hybrid proteins were immunoprecipitated with either anti-lysostaphin (1-3) or anti-enterotoxin B (4-5), separated on 12% SDS-PAGE and fluorographed.

These results provide evidence that lysostaphin is specifically addressed to its target organism *S. aureus*. The information for target cell specificity is encoded within the C-terminal 92 residues of lysostaphin. Because *S. simulans* and *S. aureus* cells have a similar peptidoglycan structure (K.H. Schleifer & O. Kandler (1972), supra) it is likely that the targeting domain of lysostaphin recognizes a non-peptidoglycan component of

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the *S. aureus* cell wall. The targeting mechanism is not required for the enzymatic activity of lysostaphin because a mutant bacteriocin truncated for its targeting signal retained activity, although it had lost the ability to specifically kill *S. aureus* cells. These results also suggest that the targeting mechanism may be responsible, at least in part, for the relative immunity of *S. simulans* to lysostaphin even though the pentaglycine crossbridges in the peptidoglycan of *S. simulans* can be cleaved by lysostaphin. Only when incubated in the presence of large amounts of enzyme can the peptidoglycan of *S. simulans* be completely digested with lysostaphin (P. Heinrich et al., "The Molecular Organization of the Lysostaphin Gene and Its Sequences Repeated in Tandem," Mol. Gen. Genet. 209: 563-569 (1987)), indicating that the cell wall of the host organism does serve as a substrate of bacteriocin activity.

Recently, Sloan and co-workers proposed a hypothesis for the immunity of *S. simulans* to the secreted lysostaphin (H.P. Dehart et al., "The Lysostaphin Endopeptidase Resistance Gene (*epr*) Specifies Modification of Peptidoglycan Cross Bridges in *Staphylococcus simulans* and *Staphylococcus aureus*," Appl. Environ. Microbiol. 61: 1475-1479 (1995)). When *S. simulans* is cured of the plasmid encoding the lysostaphin gene, it displays an increased sensitivity to lysostaphin, similar to that of *S. aureus* cells (H.E. Heath et al., "Plasmid-Encoded Lysostaphin Endopeptidase Resistance of *Staphylococcus simulans* biovar *staphylolyticus*," Biochem. Biophys. Res. Comm. 160: 1106-

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1109 (1989)). Cloning of the lysostaphin gene and its flanking DNA sequences into *S. aureus* resulted in viable cells that were less sensitive to lysostaphin digestion, suggesting that at least two genes specifying for

5 lysostaphin and a proposed immunity determinant (*epr*) were present. Compositional peptidoglycan analysis suggested that both wild-type *S. simulans* as well as *epr* transformed *S. aureus* contain an increased amount of serine over glycine residues (Dehart et al. (1995),

10 supra). The authors proposed that an altered peptidoglycan structure, for example a replacement of glycine with serine in the wall crossbridge, may be responsible for the relative immunity of *S. simulans* to secreted lysostaphin. Another interpretation of this

15 data would be that the *epr* determinant functions to alter the bacterial envelope so that it cannot be recognized by the targeting signal of lysostaphin. This hypothesis would certainly be consistent with the well established fact that the cell wall of *S. simulans* can be digested

20 with lysostaphin.

Jayaswal and co-workers reported the homology of C-terminal sequences between lysostaphin and staphylococcal phage amidase (A. Wang et al. (1991), supra). The results here demonstrate that the C-terminal

25 sequence of staphylococcal amidase also functions as a targeting signal to direct proteins to the staphylococcal cell wall. A database search identified homologies between the targeting signal of lysostaphin and C-terminal sequences of cell wall-associated proteins of

30 other Gram-positive bacteria. Other cell wall-associated proteins, such as peptidoglycan hydrolase from

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pneumococci (E. Diaz et al., "Subcellular Localization of the Major Pneumococcal Autolysin: A Peculiar Mechanism of Secretion in *Escherichia coli*," J. Biol. Chem. 264: 1238-1244 (1989)), have similar modular organization. The C-terminal repeat domains of *Streptococcus pneumoniae* amidase, for example, promote its binding to choline within the pneumococcal cell wall (J.M. Sanz et al., "Studies on the Structure and Function of the N-Terminal Domain of the Pneumococcal Murein Hydrolases," Mol. Microbiol. 6: 921-931 (1992)), a compound that is not present in staphylococcal peptidoglycans. The lysostaphin receptor from the cell wall of *S. aureus* is currently being purified. The reported measurements of  $10^6$  lysostaphin binding sites per colony forming unit suggest that the lysostaphin receptor may not be a protein molecule.

Because the bacterial cell wall is a complex organelle requiring both concerted assembly and turnover at specific sites, it is plausible that targeting domains of enzymes responsible for such processes direct the molecules to their specific locations within the cell wall. For example, the localized hydrolysis and synthesis of peptidoglycan during cell division at the newly forming septum requires enzymes to be properly addressed to this site (T. Oshida et al., "A *Staphylococcus aureus* Autolysin That Has an N-Acetylmuramoyl-L-Alanine Amidase Domain and an Endo- $\beta$ -N-Acetylglucosaminidase Domain: Cloning, Sequence Analysis, and Characterization," Proc. Natl. Acad. Sci. USA 92: 285-289 (1995); S. Yamada et al., "An Autolysin Ring Associated with Cell Separation of *Staphylococcus*



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aureus," J. Bacteriol. 178: 1565-1571 (1996)). The molecular information for these events is likely encoded in both the targeted polypeptide chains as well as the specific chemical nature of a distinct site within the cell wall.

#### ADVANTAGES OF THE PRESENT INVENTION

The processes and compositions of the present invention allow the specific noncovalent targeting of a wide variety of molecules to the cell surfaces of Gram-positive bacteria, particularly *Staphylococcus aureus*. The molecules targeted can be used for the generation of vaccines by immunization, the screening of cloned genes in a manner similar to that for phage display, and for the targeting of antibiotics and labeling substances to pathogenic bacteria. Large protein molecules can be accommodated as long as the chimeric protein can be formed. In addition, non-protein molecules such as alkaloids, steroids, and carbohydrates can be bound to the cell surface by covalent linkage to a peptide containing the cell-wall targeting signal. The cell-wall targeting produced by the process of the present invention is stable, and the resulting targeted molecules are accessible to the medium for reaction with ligands, such as molecules of the immune system that recognize antigens. A wider variety of molecules can be targeted than is the case for phage display, which is limited to relatively small molecules. This method provides great versatility, and has utility in the preparation of vaccines, the detection of cloned proteins in surface display in biotechnology, and in the specific targeting

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of antibiotics and labeling molecules to pathogenic gram-positive bacteria.

Although the present invention has been described with considerable detail, with reference to  
5 certain preferred versions thereof, other versions and embodiments are possible.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION

(i) APPLICANTS: Schneewind, Olaf  
Baba, Tadashi

(ii) TITLE OF THE INVENTION: TARGETING OF PROTEINS TO THE CELL  
WALL OF GRAM-POSITIVE BACTERIA

(iii) NUMBER OF SEQUENCES: 11

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## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: FastSEQ Version 1.5

## (vi) CURRENT APPLICATION DATA:

(A) INTERNATIONAL APPLICATION NUMBER:  
(B) FILING DATE: 22-AUG-1996  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: \_\_\_\_\_  
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(C) APPLICATION NUMBER: 60/002,615  
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-74-

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 92 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Trp	Lys	Thr	Asn	Lys	Tyr	Gly	Thr	Leu	Tyr	Lys	Ser	Glu	Ser	Ala	Ser
1				5					10					15	
Phe	Thr	Pro	Asn	Thr	Asp	Ile	Ile	Thr	Arg	Thr	Thr	Gly	Pro	Phe	Arg
			20					25					30		
Ser	Met	Pro	Gln	Ser	Gly	Val	Leu	Lys	Ala	Gly	Gln	Thr	Ile	His	Tyr
		35					40				45				
Asp	Glu	Val	Met	Lys	Gln	Asp	Gly	His	Val	Trp	Val	Gly	Tyr	Tyr	Gly
	50				55						60				
Asn	Ser	Gly	Gln	Arg	Ile	Tyr	Leu	Pro	Val	Arg	Thr	Trp	Asn	Lys	Ser
65				70						75				80	
Thr	Asn	Thr	Leu	Gly	Val	Leu	Trp	Gly	Thr	Ile	Lys				
			85							90					

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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 99 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Ala Ser Ala Trp Lys Arg Asn Lys Tyr Gly Thr Tyr Tyr Met Glu  
1 5 10 15  
Glu Ser Ala Arg Phe Thr Asn Gly Asn Gln Pro Ile Thr Val Arg Lys  
20 25 30  
Val Gly Pro Phe Leu Ser Cys Pro Val Gly Tyr Gln Phe Gln Pro Gly  
35 40 45  
Gly Tyr Cys Asp Tyr Thr Glu Val Met Leu Asn Asp Gly His Val Trp  
50 55 60  
Val Gly Tyr Thr Trp Glu Gly Gln Arg Tyr Tyr Leu Pro Ile Arg Thr  
65 70 75 80  
Trp Asn Gly Ser Ala Pro Pro Asn Gln Ile Leu Gly Asp Leu Trp Gly  
85 90 95  
Glu Ile Ser

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 amino acids
- (B) TYPE: amino acid

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(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Thr	Gly	Val	Leu	Pro	Asn	Asn	Ala	Thr	Ile	Lys	Tyr	Asp	Gly	Ala	Tyr
1				5					10					15	
Cys	Ile	Asn	Gly	Tyr	Arg	Trp	Ile	Thr	Tyr	Ile	Ala	Asn	Ser	Gly	Gln
			20					25					30		
Arg	Arg	Tyr	Ile	Ala	Thr	Gly	Glu	Val	Asp	Lys	Ala	Gly	Asn	Arg	Ile
			35				40						45		

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ser Ser Pro Thr Gln Phe Thr Phe Asn Lys Gly Glu Ser Ile Tyr Tyr

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1                      5                      10                      15  
Asp Ser Ile Leu Asn Ala Asp Gly His Gln Trp Ile Ser Tyr Arg Ser  
                    20                      25                      30  
Tyr Ser Gly Ile Arg Arg Tyr Ile  
                    35                      40

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AACATATGAA GAAAACAAAA AACAATTATT ATA

33

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

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(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGGATCCTC ACTTTATAGT TCCCCAAA

28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCCCCGGGC ATATGAAACC CTCCGATATT ACAGTT

36

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA



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(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAGAATTCCA TATCTCTAAT TCATTTTAC TAA

33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Glu Val Glu Thr Ser Lys Ala Pro Val Glu Asn Thr

1

5

10

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

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(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Pro Xaa Thr Gly

1

5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Pro Glu Thr Gly

1

5

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CLAIMS

What is claimed is:

1. A method for targeting a protein to the  
5 cell wall of a Gram-positive bacterium comprising:

(a) cloning a nucleic acid segment encoding a  
chimeric protein into a Gram-positive bacterium to  
generate a cloned chimeric protein including therein a  
carboxyl-terminal cell-wall targeting signal;

10 (b) growing the bacterium into which the  
nucleic acid segment has been cloned to express the  
cloned chimeric protein in the Gram-positive bacterium to  
generate a chimeric protein including therein a carboxyl-  
terminal cell-wall targeting signal; and

15 (c) binding the expressed chimeric protein  
noncovalently and stably to the cell-wall via the  
carboxyl-terminal cell-wall targeting signal so that the  
chimeric protein is displayed on the surface of the Gram-  
positive bacterium in such a way that the protein is  
20 accessible to a ligand.

2. The method of claim 1 wherein the carboxyl-  
terminal cell-wall targeting signal is: (a) a 92-amino-  
acid signal derived from the carboxyl-terminal sequence  
25 of the lysostaphin gene of *Staphylococcus simulans*; (b) a  
99-amino-acid signal derived from the carboxyl-terminal  
sequence of the amidase gene of *Staphylococcus aureus*;  
(c) a signal related to the signals of (a) or (b) by one  
or more conservative amino acid substitutions that  
30 preserve the existence of consensus sequence regions

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occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-  
5 A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total  
10 length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.

15 3. The method of claim 2 wherein the carboxyl-terminal cell-wall targeting signal is the lysostaphin signal having an amino acid sequence of W-K-T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-W-V-G-Y-Y-G-  
20 N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-L-W-G-T-I-K (SEQ ID NO: 1).

4. The method of claim 2 wherein the carboxyl-terminal cell-wall targeting signal is the amidase signal  
25 having an amino acid sequence of V-A-S-A-W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

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5. The method of claim 2 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of  
5 consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

6. The method of claim 5 wherein the carboxyl-terminal cell-wall targeting signal has the sequence W-K-  
10 X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>, wherein the residues designated by  
15 subscripts are chosen so that the conformation of the targeting signal remains substantially equivalent to that of the lysostaphin or amidase targeting signals.

7. The method of claim 6 wherein X<sub>3</sub> is R or T,  
20 X<sub>9</sub> is Y or L, X<sub>11</sub> is M or K, X<sub>12</sub> is E or S, X<sub>16</sub> is R or S, X<sub>19</sub> is N or P, X<sub>20</sub> is G or N, X<sub>21</sub> is N or T, X<sub>22</sub> is Q or D, X<sub>23</sub> is P or I, X<sub>26</sub> is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X<sub>28</sub> is K or T, X<sub>29</sub> is V or T, X<sub>33</sub> is L  
25 or R, X<sub>35</sub> is G or M, X<sub>37</sub> is V or Q, X<sub>38</sub> is G or S, X<sub>39</sub> is Y or G, X<sub>40</sub> is Q or V, X<sub>41</sub> is F or L, X<sub>42</sub> is Q or K, X<sub>43</sub> is P or A, X<sub>45</sub> is G or Q, X<sub>46</sub> is Y or T, X<sub>47</sub> is C or I, X<sub>48</sub> is D or H, X<sub>50</sub> is T or D, X<sub>54</sub> is L or K, X<sub>65</sub> is W or G, X<sub>66</sub> is E or N, X<sub>67</sub> is an absent amino acid with an amide bond  
30 between the preceding and following amino acids in the

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chain or S,  $X_{71}$  is Y or I,  $X_{80}$  is G or K,  $X_{82}$  is A or T,  $X_{83}$  is P or N,  $X_{84}$  is P or T,  $X_{85}$  is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{86}$  is Q or an absent amino acid  
5 with an amide bond between the preceding and following amino acids in the chain,  $X_{87}$  is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{94}$  is E or T, and  $X_{96}$  is S or K.

10 8. The method of claim 5 wherein:

(a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice  
15 versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and

(b) the conservative amino acid substitutions in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other  
20 amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for  
25 arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

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9. The method of claim 1 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

10. The method of claim 1 wherein the Gram-positive bacterium is a *Staphylococcus* species.

11. The method of claim 10 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

12. A method for targeting a protein to the cell wall of a Gram-positive bacterium comprising:

(a) generating a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal by expression of a nucleotide sequence encoding the chimeric protein in an expression system other than an expression system employing a Gram-positive bacterium as host;

(b) adding the chimeric protein to a culture medium in which a Gram-positive bacterium is growing; and

(c) allowing the chimeric protein to bind stably and noncovalently to the cell wall of the Gram-positive bacterium so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand..

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13. The method of claim 12 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.

25

14. The method of claim 13 wherein the carboxyl-terminal cell-wall targeting signal is the lysostaphin signal having an amino acid sequence of W-K-T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-

30



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W-V-G-Y-Y-G-N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-  
L-W-G-T-I-K (SEQ ID NO: 1).

15. The method of claim 13 wherein the  
5 carboxyl-terminal cell-wall targeting signal is the  
amidase signal having an amino acid sequence of V-A-S-A-  
W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-  
V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-  
G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-  
10 Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

16. The method of claim 13 wherein the  
carboxyl-terminal cell-wall targeting signal is a signal  
related to the lysostaphin signal by one or more  
15 conservative amino acid substitutions that preserve the  
existence of consensus sequence regions occurring in both  
the lysostaphin signal and the amidase signal.

17. The method of claim 16 wherein the  
20 carboxyl-terminal cell-wall targeting signal has the  
sequence W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-  
X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-  
X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-  
Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-  
25 X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>, wherein the residues  
designated by subscripts are chosen so that the  
conformation of the targeting signal remains  
substantially equivalent to that of the lysostaphin or  
amidase targeting signals.

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18. The method of claim 17 wherein  $X_3$  is R or T,  $X_9$  is Y or L,  $X_{11}$  is M or K,  $X_{12}$  is E or S,  $X_{16}$  is R or S,  $X_{19}$  is N or P,  $X_{20}$  is G or N,  $X_{21}$  is N or T,  $X_{22}$  is Q or D,  $X_{23}$  is P or I,  $X_{26}$  is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{28}$  is K or T,  $X_{29}$  is V or T,  $X_{33}$  is L or R,  $X_{35}$  is G or M,  $X_{37}$  is V or Q,  $X_{38}$  is G or S,  $X_{39}$  is Y or G,  $X_{40}$  is Q or V,  $X_{41}$  is F or L,  $X_{42}$  is Q or K,  $X_{43}$  is P or A,  $X_{45}$  is G or Q,  $X_{46}$  is Y or T,  $X_{47}$  is C or I,  $X_{48}$  is D or H,  $X_{50}$  is T or D,  $X_{54}$  is L or K,  $X_{65}$  is W or G,  $X_{66}$  is E or N,  $X_{67}$  is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S,  $X_{71}$  is Y or I,  $X_{80}$  is G or K,  $X_{82}$  is A or T,  $X_{83}$  is P or N,  $X_{84}$  is P or T,  $X_{85}$  is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{86}$  is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{87}$  is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{94}$  is E or T, and  $X_{96}$  is S or K.

19. The method of claim 16 wherein:

- (a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and
- (b) the conservative amino acid substitutions in less highly conserved regions are the following: any

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of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

20. The method of claim 12 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

21. The method of claim 12 wherein the Gram-positive bacterium is a *Staphylococcus* species.

22. The method of claim 21 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

23. A method for producing a vaccine to an antigen comprising immunizing an antibody-producing animal with a complex comprising a chimeric protein including therein: (1) a protein antigen and (2) a

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carboxyl-terminal cell wall-targeting signal bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is  
5 displayed on the surface of the Gram-positive bacterium in such a way that the chimeric protein is accessible to the antigen-processing system of the antibody-producing animal.

10 24. The method of claim 23 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the  
15 carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the  
20 lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID  
25 NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is  
30 reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal

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results in at least about 80% homology and at least about 70% identity.

25. The method of claim 24 wherein the  
5 carboxyl-terminal cell-wall targeting signal is the  
lysostaphin signal having an amino acid sequence of W-K-  
T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-  
F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-  
W-V-G-Y-Y-G-N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-  
10 L-W-G-T-I-K (SEQ ID NO: 1).

26. The method of claim 24 wherein the  
carboxyl-terminal cell-wall targeting signal is the  
amidase signal having an amino acid sequence of V-A-S-A-  
15 W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-  
V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-  
G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-  
Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

20 27. The method of claim 23 wherein the  
carboxyl-terminal cell-wall targeting signal is a signal  
related to the lysostaphin signal by one or more  
conservative amino acid substitutions that preserve the  
existence of consensus sequence regions occurring in both  
25 the lysostaphin signal and the amidase signal.

28. The method of claim 27 wherein the  
carboxyl-terminal cell-wall targeting signal has the  
sequence W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-  
30 X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-

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X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>, wherein the residues designated by subscripts are chosen so that the

5 conformation of the targeting signal remains substantially equivalent to that of the lysostaphin or amidase targeting signals.

29. The method of claim 28 wherein X<sub>3</sub> is R or T, X<sub>9</sub> is Y or L, X<sub>11</sub> is M or K, X<sub>12</sub> is E or S, X<sub>16</sub> is R or S, X<sub>19</sub> is N or P, X<sub>20</sub> is G or N, X<sub>21</sub> is N or T, X<sub>22</sub> is Q or D, X<sub>23</sub> is P or I, X<sub>26</sub> is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X<sub>28</sub> is K or T, X<sub>29</sub> is V or T, X<sub>33</sub> is L or R, X<sub>35</sub> is G or M, X<sub>37</sub> is V or Q, X<sub>38</sub> is G or S, X<sub>39</sub> is Y or G, X<sub>40</sub> is Q or V, X<sub>41</sub> is F or L, X<sub>42</sub> is Q or K, X<sub>43</sub> is P or A, X<sub>45</sub> is G or Q, X<sub>46</sub> is Y or T, X<sub>47</sub> is C or I, X<sub>48</sub> is D or H, X<sub>50</sub> is T or D, X<sub>54</sub> is L or K, X<sub>65</sub> is W or G, X<sub>66</sub> is E or N, X<sub>67</sub> is an absent amino acid with an amide bond

15 between the preceding and following amino acids in the chain or S, X<sub>71</sub> is Y or I, X<sub>80</sub> is G or K, X<sub>82</sub> is A or T, X<sub>83</sub> is P or N, X<sub>84</sub> is P or T, X<sub>85</sub> is N or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X<sub>86</sub> is Q or an absent amino acid with an amide bond between the preceding and following

20 amino acids in the chain, X<sub>87</sub> is I or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X<sub>94</sub> is E or T, and X<sub>96</sub> is S or K.

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30. The method of claim 27 wherein:

(a) the conservative amino acid substitutions in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and

(b) the conservative amino acid substitutions in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

20

31. The method of claim 23 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ. ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

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32. The method of claim 23 wherein the Gram-positive bacterium is a *Staphylococcus* species.

33. The method of claim 32 wherein the  
5 *Staphylococcus* species is *Staphylococcus aureus*.

34. The method of claim 23 wherein the antigen is a polypeptide antigen produced by *Candida albicans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*,  
10 *Microsporium canis*, *Plasmodium falciparum*, *Trypanosoma cruzi*, *Borrelia bergdorferi*, *Treponema pallidum*, *Borrelia recurrentis*, *Leptospira icterohaemorrhagiae*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhosa*, *Hemophilus*  
15 *influenzae*, *Bordetella pertussis*, *Actinomyces israelii*, *Streptococcus mutans*, *Streptococcus equi*, *Streptococcus agalactiae*, *Streptococcus anginosus*, human immunodeficiency virus (HIV), poliovirus, influenza virus, rabies virus, herpes virus, foot and mouth disease  
20 virus, psittacosis virus, paramyxovirus, myxovirus, or coronavirus.

35. A method for producing a vaccine to an antigen comprising immunizing an antibody-producing  
25 animal with a complex comprising a non-protein antigen or hapten covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal, the complex bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-  
30 wall targeting signal so that the complex is displayed on



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the surface of the Gram-positive bacterium in such a way that the complex is accessible to the antigen-processing system of the antibody-producing animal.

5           36. The method of claim 35 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the  
10 carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the  
15 lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID  
20 NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best  
25 alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.

30           37. The method of claim 36 wherein the carboxyl-terminal cell-wall targeting signal is the

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lysostaphin signal having an amino acid sequence of W-K-T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-W-V-G-Y-Y-G-N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-L-W-G-T-I-K (SEQ ID NO: 1).

38. The method of claim 36 wherein the carboxyl-terminal cell-wall targeting signal is the amidase signal having an amino acid sequence of V-A-S-A-W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

39. The method of claim 35 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

40. The method of claim 39 wherein the carboxyl-terminal cell-wall targeting signal has the sequence W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>, wherein the residues designated by subscripts are chosen so that the conformation of the targeting signal remains

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substantially equivalent to that of the lysostaphin or amidase targeting signals.

41. The method of claim 40 wherein  $X_3$  is R or  
5 T,  $X_9$  is Y or L,  $X_{11}$  is M or K,  $X_{12}$  is E or S,  $X_{16}$  is R or  
S,  $X_{19}$  is N or P,  $X_{20}$  is G or N,  $X_{21}$  is N or T,  $X_{22}$  is Q or  
D,  $X_{23}$  is P or I,  $X_{26}$  is V or an absent amino acid with an  
amide bond between the preceding and following amino  
acids in the chain,  $X_{28}$  is K or T,  $X_{29}$  is V or T,  $X_{33}$  is L  
10 or R,  $X_{35}$  is G or M,  $X_{37}$  is V or Q,  $X_{38}$  is G or S,  $X_{39}$  is Y  
or G,  $X_{40}$  is Q or V,  $X_{41}$  is F or L,  $X_{42}$  is Q or K,  $X_{43}$  is P  
or A,  $X_{45}$  is G or Q,  $X_{46}$  is Y or T,  $X_{47}$  is C or I,  $X_{48}$  is D  
or H,  $X_{50}$  is T or D,  $X_{54}$  is L or K,  $X_{65}$  is W or G,  $X_{66}$  is E  
or N,  $X_{67}$  is an absent amino acid with an amide bond  
15 between the preceding and following amino acids in the  
chain or S,  $X_{71}$  is Y or I,  $X_{80}$  is G or K,  $X_{82}$  is A or T,  $X_{83}$   
is P or N,  $X_{84}$  is P or T,  $X_{85}$  is N or an absent amino acid  
with an amide bond between the preceding and following  
amino acids in the chain,  $X_{86}$  is Q or an absent amino acid  
20 with an amide bond between the preceding and following  
amino acids in the chain,  $X_{87}$  is I or an absent amino acid  
with an amide bond between the preceding and following  
amino acids in the chain,  $X_{94}$  is E or T, and  $X_{96}$  is S or K.

25 42. The method of claim 39 wherein:

(a) the conservative amino acid substitutions  
in highly conserved regions are the following: any of  
isoleucine, valine, and leucine for any of these other  
amino acids; aspartic acid for glutamic acid and vice

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versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and

(b) the conservative amino acid substitutions in less highly conserved regions are the following: any  
5 of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine  
10 for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

15

43. The method of claim 35 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and includes therein an amino acid sequence that is T-G-V-L-  
20 P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

25

44. The method of claim 35 wherein the Gram-positive bacterium is a *Staphylococcus* species.

45. The method of claim 44 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

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46. The method of claim 35 wherein the antigen or hapten is a drug, an alkaloid, a steroid, a carbohydrate, or an aromatic compound.

5           47. A method for producing a vaccine to an antigen comprising immunizing an antibody-producing animal with a complex comprising a protein antigen covalently linked to a protein including therein a carboxyl-terminal cell wall-targeting signal, the complex  
10 bound noncovalently and stably to the cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal so that the complex is displayed on the surface of the Gram-positive bacterium in such a way that the complex is accessible to the antigen-processing  
15 system of the antibody-producing animal.

          48. The method of claim 47 wherein the carboxyl-terminal cell-wall targeting signal is: (a) a  
carboxyl-terminal cell-wall targeting signal is: (a) a  
92-amino-acid signal derived from the carboxyl-terminal  
20 sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of  
*Staphylococcus aureus*; (c) a signal related to the signals of (a) or (b) by one or more conservative amino  
25 acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino acids, and that includes therein an amino acid sequence  
30 that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-

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T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e) a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about 70% identity.

10

49. The method of claim 48 wherein the carboxyl-terminal cell-wall targeting signal is the lysostaphin signal having an amino acid sequence of W-K-T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-W-V-G-Y-Y-G-N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-L-W-G-T-I-K (SEQ ID NO: 1).

50. The method of claim 48 wherein the carboxyl-terminal cell-wall targeting signal is the amidase signal having an amino acid sequence of V-A-S-A-W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

51. The method of claim 47 wherein the carboxyl-terminal cell-wall targeting signal is a signal related to the lysostaphin signal by one or more conservative amino acid substitutions that preserve the

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existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

52. The method of claim 51 wherein the  
5 carboxyl-terminal cell-wall targeting signal has the  
sequence W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-  
X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-  
X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-  
Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-  
10 X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>, wherein the residues  
designated by subscripts are chosen so that the  
conformation of the targeting signal remains  
substantially equivalent to that of the lysostaphin or  
amidase targeting signals.

15

53. The method of claim 52 wherein X<sub>3</sub> is R or  
T, X<sub>9</sub> is Y or L, X<sub>11</sub> is M or K, X<sub>12</sub> is E or S, X<sub>16</sub> is R or  
S, X<sub>19</sub> is N or P, X<sub>20</sub> is G or N, X<sub>21</sub> is N or T, X<sub>22</sub> is Q or  
D, X<sub>23</sub> is P or I, X<sub>26</sub> is V or an absent amino acid with an  
20 amide bond between the preceding and following amino  
acids in the chain, X<sub>28</sub> is K or T, X<sub>29</sub> is V or T, X<sub>33</sub> is L  
or R, X<sub>35</sub> is G or M, X<sub>37</sub> is V or Q, X<sub>38</sub> is G or S, X<sub>39</sub> is Y  
or G, X<sub>40</sub> is Q or V, X<sub>41</sub> is F or L, X<sub>42</sub> is Q or K, X<sub>43</sub> is P  
or A, X<sub>45</sub> is G or Q, X<sub>46</sub> is Y or T, X<sub>47</sub> is C or I, X<sub>48</sub> is D  
25 or H, X<sub>50</sub> is T or D, X<sub>54</sub> is L or K, X<sub>65</sub> is W or G, X<sub>66</sub> is E  
or N, X<sub>67</sub> is an absent amino acid with an amide bond  
between the preceding and following amino acids in the  
chain or S, X<sub>71</sub> is Y or I, X<sub>80</sub> is G or K, X<sub>82</sub> is A or T, X<sub>83</sub>  
is P or N, X<sub>84</sub> is P or T, X<sub>85</sub> is N or an absent amino acid  
30 with an amide bond between the preceding and following

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amino acids in the chain,  $X_{86}$  is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{87}$  is I or an absent amino acid with an amide bond between the preceding and following  
5 amino acids in the chain,  $X_{94}$  is E or T, and  $X_{96}$  is S or K.

54. The method of claim 51 wherein:

(a) the conservative amino acid substitutions in highly conserved regions are the following: any of  
10 isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and

(b) the conservative amino acid substitutions  
15 in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine for threonine and vice versa; glycine for alanine and  
20 vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of arginine or lysine; glutamine for glutamic acid and vice  
25 versa; and asparagine for aspartic acid and vice versa.

55. The method of claim 47 wherein the carboxyl-terminal cell wall targeting signal has from about 80 amino acids to about 100 amino acids, and  
30 includes therein an amino acid sequence that is T-G-V-L-



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P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

5

56. A method for screening a cloned protein for reactivity with a specific binding partner comprising:

(a) incorporating a cloned protein into a chimeric protein including therein a carboxyl-terminal cell wall-targeting signal;

(b) binding the chimeric protein to the surface of a Gram-positive bacterium to form a stable noncovalent complex; and

(c) reacting the noncovalent complex with a labeled specific binding partner to screen the cloned protein for reactivity with the specific binding partner.

57. The method of claim 56 wherein the labeled specific binding partner is an antibody.

58. A method for treating an infection caused by a Gram-positive bacterium comprising:

(a) conjugating an antibiotic to a protein including therein a carboxyl-terminal cell wall-targeting signal to produce a conjugate; and

(b) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the conjugate noncovalently to the cell walls of the bacterium in order to treat the infection.

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59. The method of claim 58 wherein the antibiotic is a penicillin, ampicillin, vancomycin, gentamicin, streptomycin, a cephalosporin, amikacin, 5 kanamycin, neomycin, paromomycin, tobramycin, ciprofloxacin, clindamycin, rifampin, chloramphenicol, or norfloxacin.

60. The method of claim 58 wherein the Gram- 10 positive bacterium is a *Staphylococcus* species.

61. The method of claim 60 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

15 62. A method for detecting the presence of a Gram-positive bacterium so as to diagnose an infection caused by a Gram-positive bacterium comprising:

(a) conjugating a diagnostic reagent to a protein including therein a carboxyl-terminal cell wall- 20 targeting signal to produce a conjugate; and

(b) introducing the conjugate to an organism infected with a Gram-positive bacterium in order to bind the diagnostic reagent noncovalently to the cell walls of the bacterium, thereby detecting the Gram-positive 25 bacterium and diagnosing the infection.

63. The method of claim 62 wherein the diagnostic reagent is an antibody labeled with a detectable label.

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64. The method of claim 62 wherein the Gram-positive bacterium is a *Staphylococcus* species.

65. The method of claim 64 wherein the  
5 *Staphylococcus* species is *Staphylococcus aureus*.

66. A chimeric protein comprising:

(a) a protein to be targeted to the cell wall of a Gram-positive bacterium; and

10 (b) a cell-wall targeting signal located at the carboxyl terminus of the chimeric protein for binding the chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the  
15 surface of the Gram-positive bacterium in such a way that the protein is accessible to a ligand.

67. The chimeric protein of claim 66 wherein the carboxyl-terminal cell-wall targeting signal is: (a)  
20 a 92-amino-acid signal derived from the carboxyl-terminal sequence of the lysostaphin gene of *Staphylococcus simulans*; (b) a 99-amino-acid signal derived from the carboxyl-terminal sequence of the amidase gene of *Staphylococcus aureus*; (c) a signal related to the  
25 signals of (a) or (b) by one or more conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal; (d) a signal that has from about 80 amino acids to about 100 amino  
30 acids, and that includes therein an amino acid sequence

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that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4); or (e)  
5 a signal related to the signals of (a), (b), (c), or (d) by truncation such that the total length of the signal is reduced to no less than about 80 amino acids and the best alignment of the truncated signal and the original signal results in at least about 80% homology and at least about  
10 70% identity.

68. The chimeric protein of claim 67 wherein the carboxyl-terminal cell-wall targeting signal is the lysostaphin signal having an amino acid sequence of W-K-  
15 T-N-K-Y-G-T-L-Y-K-S-E-S-A-S-F-T-P-N-T-D-I-I-T-R-T-T-G-P-F-R-S-M-P-Q-S-G-V-L-K-A-G-Q-T-I-H-Y-D-E-V-M-K-Q-D-G-H-V-W-V-G-Y-Y-G-N-S-G-Q-R-I-Y-L-P-V-R-T-W-N-K-S-T-N-T-L-G-V-L-W-G-T-I-K (SEQ ID NO: 1).

20 69. The chimeric protein of claim 67 wherein the carboxyl-terminal cell-wall targeting signal is the amidase signal having an amino acid sequence of V-A-S-A-W-K-R-N-K-Y-G-T-Y-Y-M-E-E-S-A-R-F-T-N-G-N-Q-P-I-T-V-R-K-V-G-P-F-L-S-C-P-V-G-Y-Q-F-Q-P-G-G-Y-C-D-Y-T-E-V-M-L-N-D-  
25 G-H-V-W-V-G-Y-T-W-E-G-Q-R-Y-Y-L-P-I-R-T-W-N-G-S-A-P-P-N-Q-I-L-G-D-L-W-G-E-I-S (SEQ ID NO: 2).

70. The chimeric protein of claim 67 wherein the carboxyl-terminal cell-wall targeting signal is a  
30 signal related to the lysostaphin signal by one or more

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conservative amino acid substitutions that preserve the existence of consensus sequence regions occurring in both the lysostaphin signal and the amidase signal.

5           71. The chimeric protein of claim 70 wherein the carboxyl-terminal cell-wall targeting signal has the sequence W-K-X<sub>3</sub>-N-K-T-G-T-X<sub>9</sub>-Y-X<sub>11</sub>-X<sub>12</sub>-E-S-A-X<sub>16</sub>-F-T-X<sub>19</sub>-X<sub>20</sub>-  
X<sub>21</sub>-X<sub>22</sub>-X<sub>23</sub>-I-T-X<sub>26</sub>-R-X<sub>28</sub>-X<sub>29</sub>-G-P-F-X<sub>33</sub>-S-X<sub>35</sub>-P-X<sub>37</sub>-X<sub>38</sub>-X<sub>39</sub>-X<sub>40</sub>-  
X<sub>41</sub>-X<sub>42</sub>-X<sub>43</sub>-G-X<sub>45</sub>-X<sub>46</sub>-X<sub>47</sub>-X<sub>48</sub>-Y-X<sub>50</sub>-E-V-M-X<sub>54</sub>-Q-D-G-H-V-W-V-G-  
10 Y-T-X<sub>65</sub>-X<sub>66</sub>-X<sub>67</sub>-G-Q-R-X<sub>71</sub>-Y-L-P-X<sub>75</sub>-R-T-W-N-X<sub>80</sub>-S-X<sub>82</sub>-X<sub>83</sub>-X<sub>84</sub>-  
X<sub>85</sub>-X<sub>86</sub>-X<sub>87</sub>-L-G-V-L-W-G-X<sub>94</sub>-I-X<sub>96</sub>, wherein the residues designated by subscripts are chosen so that the conformation of the targeting signal remains substantially equivalent to that of the lysostaphin or  
15 amidase targeting signals.

72. The chimeric protein of claim 71 wherein X<sub>3</sub> is R or T, X<sub>9</sub> is Y or L, X<sub>11</sub> is M or K, X<sub>12</sub> is E or S, X<sub>16</sub> is R or S, X<sub>19</sub> is N or P, X<sub>20</sub> is G or N, X<sub>21</sub> is N or T, X<sub>22</sub> is Q or D, X<sub>23</sub> is P or I, X<sub>26</sub> is V or an absent amino acid with an amide bond between the preceding and following amino acids in the chain, X<sub>28</sub> is K or T, X<sub>29</sub> is V or T, X<sub>33</sub> is L or R, X<sub>35</sub> is G or M, X<sub>37</sub> is V or Q, X<sub>38</sub> is G or S, X<sub>39</sub> is Y or G, X<sub>40</sub> is Q or V, X<sub>41</sub> is F or L, X<sub>42</sub> is Q or K, X<sub>43</sub> is P or A, X<sub>45</sub> is G or Q, X<sub>46</sub> is Y or T, X<sub>47</sub> is C or I, X<sub>48</sub> is D or H, X<sub>50</sub> is T or D, X<sub>54</sub> is L or K, X<sub>65</sub> is W or G, X<sub>66</sub> is E or N, X<sub>67</sub> is an absent amino acid with an amide bond between the preceding and following amino acids in the chain or S, X<sub>71</sub> is Y or I, X<sub>80</sub> is G or K, X<sub>82</sub> is A or T, X<sub>83</sub> is P or N, X<sub>84</sub> is P or T, X<sub>85</sub> is N or an absent amino acid  
20  
25  
30

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with an amide bond between the preceding and following amino acids in the chain,  $X_{86}$  is Q or an absent amino acid with an amide bond between the preceding and following amino acids in the chain,  $X_{87}$  is I or an absent amino acid  
5 with an amide bond between the preceding and following amino acids in the chain,  $X_{94}$  is E or T, and  $X_{96}$  is S or K.

73. The chimeric protein of claim 70 wherein:

- (a) the conservative amino acid substitutions  
10 in highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; and serine for threonine and vice versa; and  
15 (b) the conservative amino acid substitutions in less highly conserved regions are the following: any of isoleucine, valine, and leucine for any of these other amino acids; aspartic acid for glutamic acid and vice versa; glutamine for asparagine and vice versa; serine  
20 for threonine and vice versa; glycine for alanine and vice versa; alanine for valine and vice versa; methionine for any of leucine, isoleucine, or valine; lysine for arginine or vice versa; one of aspartic acid or glutamic acid for one of arginine or lysine; histidine for one of  
25 arginine or lysine; glutamine for glutamic acid and vice versa; and asparagine for aspartic acid and vice versa.

74. The chimeric protein of claim 66 wherein the carboxyl-terminal cell wall targeting signal has from  
30 about 80 amino acids to about 100 amino acids, and

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includes therein an amino acid sequence that is T-G-V-L-P-N-N-A-T-I-K-Y-D-G-A-Y-C-I-N-G-Y-R-W-I-T-Y-I-A-N-S-G-Q-R-R-Y-I-A-T-G-E-V-D-K-A-G-N-R-I (SEQ ID NO: 3) or S-S-P-T-Q-F-T-F-N-K-G-E-S-I-Y-Y-D-S-I-L-N-A-D-G-H-Q-W-I-S-Y-R-S-Y-S-G-I-R-R-Y-I (SEQ ID NO: 4).

75. A nucleic acid segment encoding the chimeric protein of claim 66.

10 76. The nucleic acid segment of claim 75 wherein the nucleic acid segment is DNA.

77. A vector comprising the nucleic acid segment of claim 75 operatively linked to at least one control sequence that controls the expression or regulation of the nucleic acid segment.

78. A noncovalent complex comprising:  
(a) the chimeric protein of claim 66; and  
20 (b) a Gram-positive bacterium, in which the chimeric protein is bound noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface of the Gram-positive bacterium in such a way that  
25 the protein is accessible to a ligand.

79. The noncovalent complex of claim 78 wherein the Gram-positive bacterium is a *Staphylococcus* species.

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80. The noncovalent complex of claim 79 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

5           81. A covalent conjugate comprising:

          (a) a cell-wall targeting segment including a carboxyl-terminal cell-wall targeting signal therein that can be bound noncovalently and stably to a cell wall of a Gram-positive bacterium via the carboxyl-terminal cell-wall targeting signal; and

10

          (b) an antigen or hapten conjugated covalently to the cell-wall targeting segment.

          82. The covalent conjugate of claim 81 wherein

15 the antigen or hapten is a protein antigen.

          83. The covalent conjugate of claim 81 wherein the antigen or hapten is a nonprotein antigen or hapten.

20           84. The covalent conjugate of claim 81 wherein the cell-wall targeting segment further includes an amino-terminal spacer.

          85. A noncovalent complex comprising:

25           (a) the covalent conjugate of claim 81; and

          (b) a Gram-positive bacterium stably and noncovalently binding the covalent conjugate.



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86. The noncovalent complex of claim 85 wherein the Gram-positive bacterium is a *Staphylococcus* species.

5           87. The noncovalent complex of claim 86 wherein the *Staphylococcus* species is *Staphylococcus aureus*.

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Fig. 1

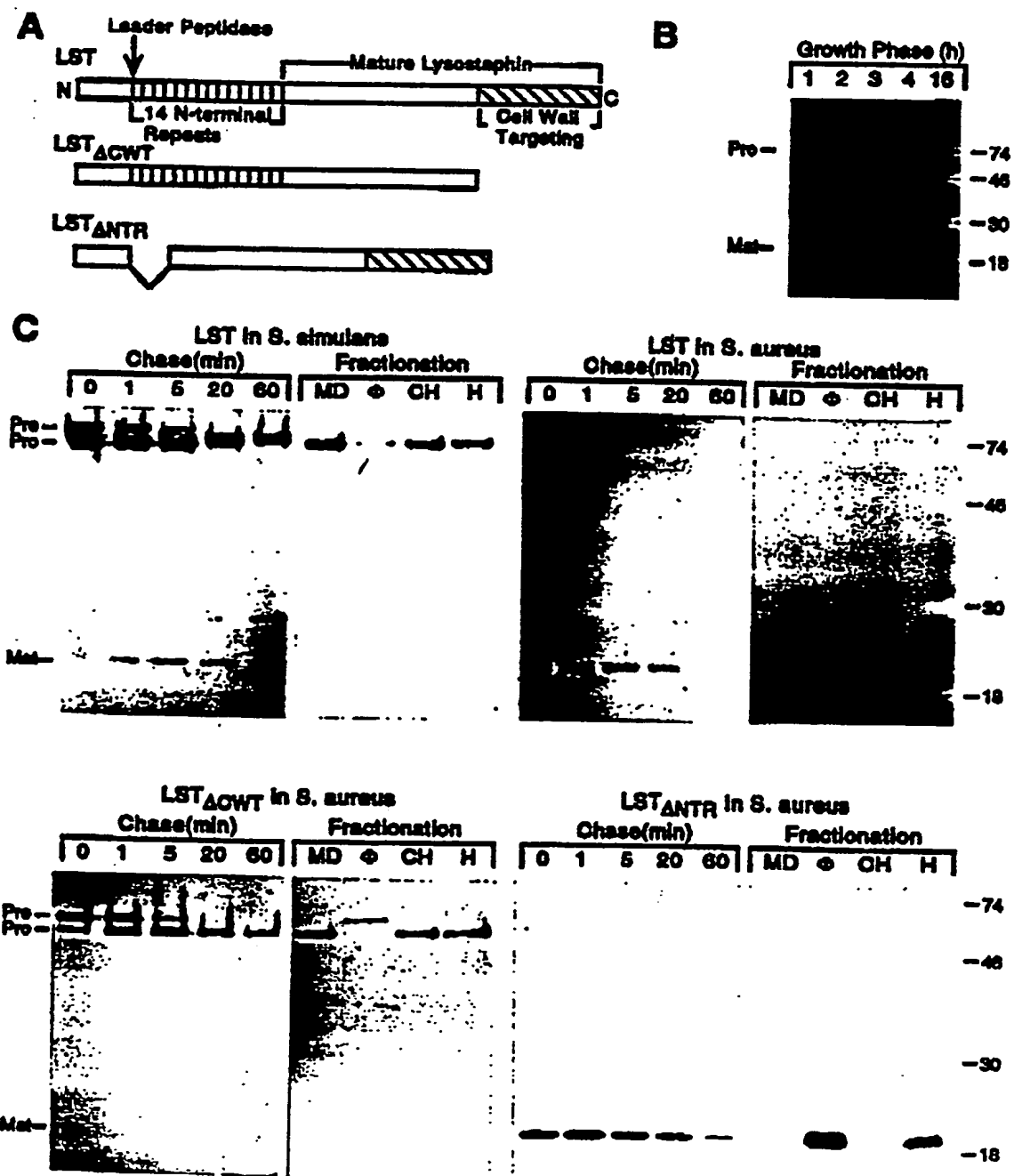
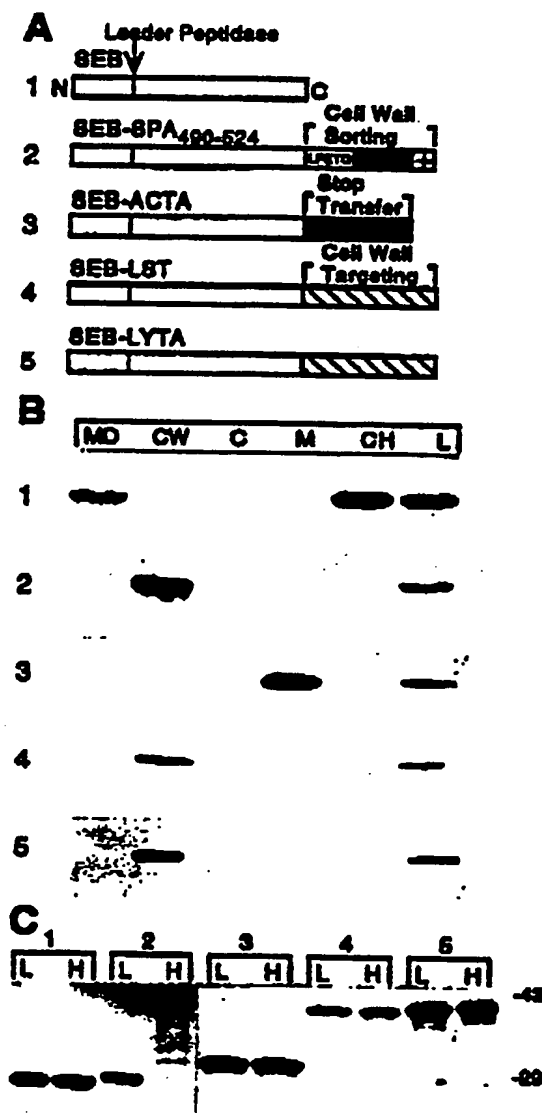


Fig. 2





## DNASIS

Maximum Matching [phill-amidase.aa VS lysostaphin.aa]

File1: phill-amidase.aa

1 - 481

File2: lysostaphin.aa

1 - 389

Matching Percentage (Total Window: 31%, Alignment Window: 31%)

```

1 MQAKLTKNEFIER-LKTSE GKQ-FNVDLW---YGF-QCFDYAN---AGWK 50
  | | | | | | | | | | | | | | | | | | | | | |
1 MK-K-TKNYYTRPLAI--GLSTFA--LASIVYGGIQ-----NETHASEK 50

51 ----VLFGLLKGLGA-----KDIPFANNFDGLA---T----VYQNTPDF 100
  | | | | | | | | | | | | | | | | | | | | | |
51 SNMDVSK-----KV--AEVETSKA-PVENT-----AEVETSKAPVE-NTAEV 100

101 L---AQPGDMV-----VFGSNYGAGYGHV---AWVIEATLDYIIVYEQNW 150
  | | | | | | | | | | | | | | | | | | | | | |
101 ETSKA-P---VENTAEVETSK--AP---VENTAEV-E-TSKAP-V-E-N- 150

151 LGGGWTDGIEQ---P---AGVGK-K-L-QDDNMLMISLCGLSVRI-LKV 200
  | | | | | | | | | | | | | | | | | | | | | |
151 -----TAEVETSKAPVENTAEVETSKALVQ--N-----RTAL-- 200

201 RQRHDQFNLLHKHPKKETAKPOPKAVELKIIKD VVKGYDLPKRGSNPKGI 250
  | | | | | | | | | | | | | | | | | | | | | |
201 RAAT-----HEHS-----A--QW-----LN NYK--KGYGY--GPYPLGI 250

251 VIHNDAGSKGATAEAYRNLV-----NA--PLSRLEAGIAHSYVSGNTVWQ 300
  | | | | | | | | | | | | | | | | | | | | | |
251 ---N--G--GMH---Y--G-VDFFMNIGTPVK---A-I--S--SGKIVE- 300

301 ALDESQVGWHTANQIGNKYYYGIE---VC-QS-MGADNATFL-K-NEQAT 350
  | | | | | | | | | | | | | | | | | | | | | |
301 AGW-SNYGGG--NQIGL-----IENDGVHROWYMH-----LSKYNV--- 350

351 FOECARLLKKWGLP--ANRNTIRLHNEFT--STSCPH-----R---S-SVL 400
  | | | | | | | | | | | | | | | | | | | | | |
351 -----KV-GDYVKAGQI-IGWSGS-TGYSTA-PHLHFQRMVNSFSNS 400

401 HTGFDPVTRGLLPEDKRLQLKDYFIKQIRA-YMDGKIPVA--TVSNESSA 450
  | | | | | | | | | | | | | | | | | | | | | |
401 -TAQDPM-----PF---L--KS-----AGY--GK---AGGTVTPTP-- 450

451 SSNTV10KPVASAWKRNKYGTYYMEESARFTNG20NOPI-TVRKV30GF40FLSCP-- 500
  | | | | | | | | | | | | | | | | | | | | | |
451 --NTG-----WKTNKYGTLYKSESASFT50P-NTDIIT-RTTGPF60RSMPQS 500

501 -V---GY60QPGGYCDYTEV70MLQDGHVW80GYTWE-GORY90YLP100IRTWNGSA 550
  | | | | | | | | | | | | | | | | | | | | | |
501 GVLKAG-OTIH--Y-D--EVMKODGHVW110GYTGN120SGORI130YLP140VRTW150NKST 550

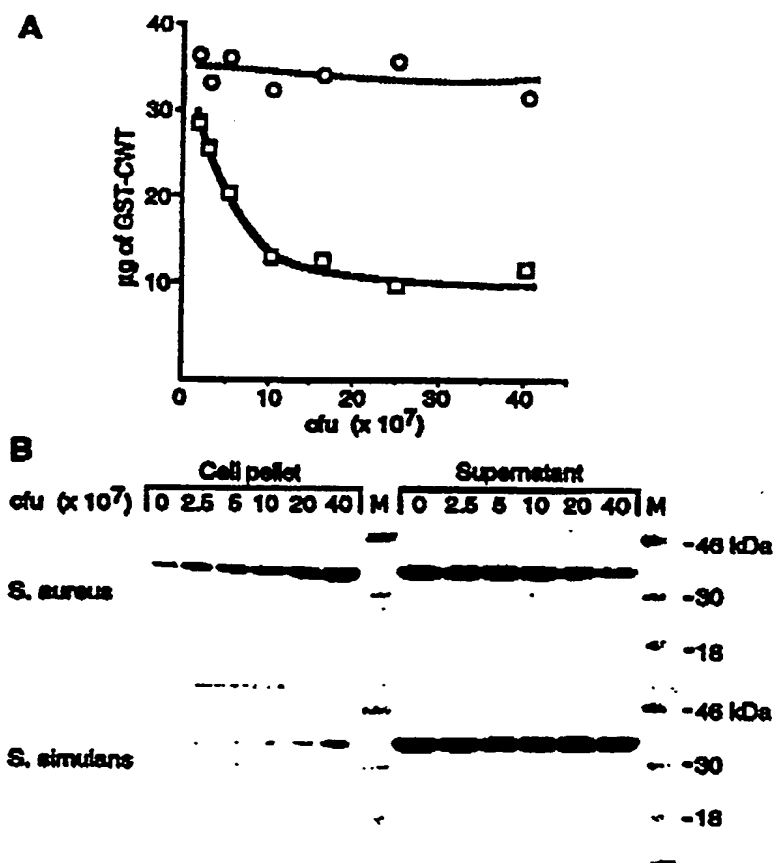
551 PPNCILGDLWGEIS'..... 600
  | | | | | | | | | | | | | | | | | | | | | |
551 --NT-LGV160LWGTIK..... 600

```

r 92

FIG. 4

Fig. 5



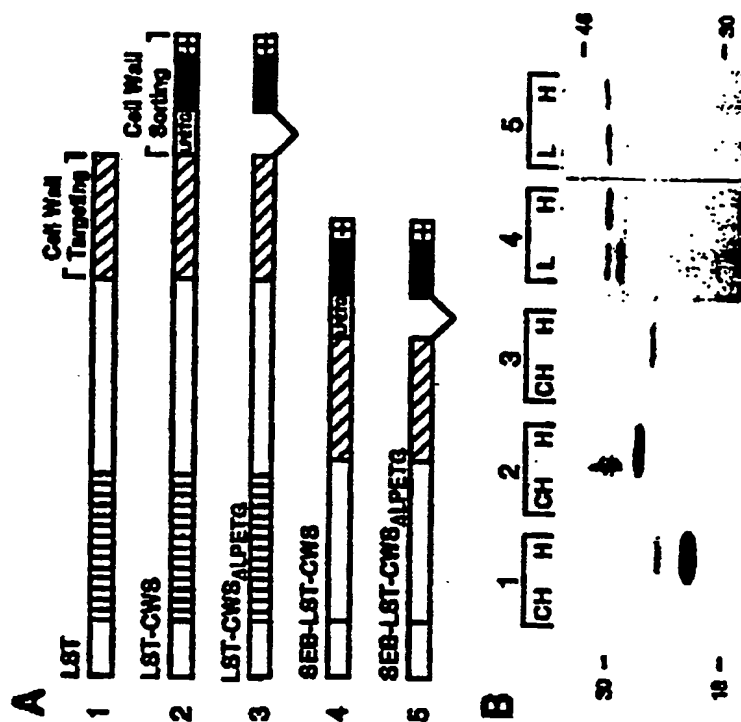
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Target Cell Specificity and Bacteriolytic Activity of  
Lysostaphin

	<u>Substrate</u>			Target Specificity
	<i>S. simulans</i>	<i>S. aureus</i>	Acetyl-Gly <sub>6</sub>	
Mock	$3.8 \times 10^5$	$4.0 \times 10^5$	0	
Mature LST	$2.1 \times 10^5$	4	$3.86 \times 10^4$	$5.3 \times 10^4$
Pro-LST	$3.1 \times 10^5$	$5.5 \times 10^2$	$3.58 \times 10^4$	$5.6 \times 10^2$
Pro-LST <sub>ΔCWT</sub>	$3.1 \times 10^5$	$2.4 \times 10^5$	$3.23 \times 10^4$	1.3

FIGURE 6

Fig. 7





## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/14154**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350, 333, 334, 387.3; 435/320.1, 254.2, 7.1, 7.33, 7.6, 252.3; 436/536, 518, 501, 543, 532; 536/25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHINDLER, C. A. et al. Lysostaphin: A new bacteriolytic agent for the Staphylococcus. Proc. Natl. Acad. Sci. USA. 1964, Vol. 51. pages 414-421, see entire document.	1-11
Y P	NAVARRE, W. W. et al. Cell wall sorting of lipoproteins in Staphylococcus aureus J. Bacteriology. January 1996, Vol. 178. No. 2. pages 441-446, see entire document.	1-11
Y	TALAY, S. R. Domain structure and conserved epitopes of Sfb protein, the fibronectin-binding adhesin of Streptococcus pyogenes Mol. Microbiol. 1994, Vol. 13. No. 3. pages 531-539, see entire document.	1-11

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:		
*A*	document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E*	earlier document published on or after the international filing date	*X*
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*
*O*	document referring to an oral disclosure, use, exhibition or other means	
*P*	document published prior to the international filing date but later than the priority date claimed	*&*

Date of the actual completion of the international search

24 OCTOBER 1996

Date of mailing of the international search report

13 NOV 1996

Name and mailing address of the ISA/US  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/14154

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHREUDER, M. P. et al. Targeting of a heterologous protein to the cell wall of <i>Saccharomyces cerevisiae</i> . <i>Yeast</i> . 1993, Vol. 9. pages 399-409, see entire document.	1-11
Y	RECSEI, P. A. et al. Cloning, sequence, and expression of the lysostaphin gene from <i>Staphylococcus simulans</i> . <i>Proc. Natl. Acad. Sci. USA</i> . March 1987, Vol. 84. pages 1127-1131, see entire document.	1-11
Y	WANG, X. et al. Sequence analysis of a <i>Staphylococcus aureus</i> gene encoding a peptidoglycan hydrolase activity. <i>Gene</i> . 1991, Vol. 102, pages 105-109, see entire document.	1-11
A	OGIER, J. A. et al. A 40-Kilodalton cell wall protein-coding sequence upstream of the sr gene of <i>Streptococcus mutans</i> OMZ175 (serotype f). <i>Infection and Immunity</i> . May 1991, Vol. 59, No. 5, pages 1620-1626, see entire document.	1-11
Y	HEINRICH, P. et al. The molecular organization of the lysostaphin gene and its sequences repeated in tandem. <i>Mol. Gen. Genet.</i> 1987, Vol. 209, pages 563-569, see entire document.	1-11
A	RAMBUKKANA, A. et al. Identification and characterization of epitopes shared between the mycobacterial 65-Kilodalton heat shock protein and the actively secreted antigen 85 complex: Their in situ expression on the cell wall surface of <i>Mycobacterium leprae</i> . <i>Infection And Immunity</i> . November 1992, Vol. 60, No. 11, pages 4517-4527, see entire document.	1-11
Y	WO 87/06264 A1 (PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK) 22 October 1987, see entire document.	1-11

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/14154

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-11

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/14154

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

G01N 33/53, 33/569, 33/536, 33/543, 33/566, 33/549, 33/531; C12N 1/20, 15/00, 15/63, 1/14, 15/09, 15/70, 15/74;  
A61K 38/00; C07K 2/00, 4/00, 5/00, 7/00, 16/00, 17/00; C12P 21/08; C07H 21/00, 21/02, 21/04

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

530/300, 350, 333, 334, 387.3; 435/320.1, 254.2, 7.1, 7.33, 7.6, 252.3; 436/536, 518, 501, 543, 532; 536/25.3

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

IntelliGenetics, MasPar, CaPlus, WPIDS, Derwent Biotechnology Abs, Medline, CAB Abstracts, Life Sciences Collection, Dissertation Abstracts Online, FEDRIP, AGRICOLA, Oceanic Abst, EMBASE, BIOSIS PREVIEWS, PASCAL, CRIS/USDA, Wilson Appl. Sci & Tech Abs

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-11, drawn to a method for targeting a protein to the cell wall of a Gram-positive bacterium.  
Group II, claim(s) 12-22, drawn to a method for targeting a protein to the cell wall of a Gram-positive bacterium.

Group III, claim(s) 23-34, drawn to method for producing a vaccine to an antigen.

Group IV, claim(s) 35-46, drawn to method for producing a vaccine to an antigen.

Group V, claim(s) 47-55, drawn to method for producing a vaccine to an antigen.

Group VI, claim(s) 56-57, drawn to method for screening a cloned protein.

Group VII, claim(s) 58-61, drawn to method for treating infection caused by a Gram-positive bacterium.

Group VIII, claim(s) 62-65, drawn to method for detecting the presence of a Gram-positive bacterium.

Group IX, claim(s) 66-80, drawn to chimeric protein.

Group X, claim(s) 81-87, drawn to covalent conjugates.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the methods set forth in Groups I and II are both drawn to a method for targeting a protein to the cell wall of a Gram-positive bacterium, however the specific steps involved in each method are different. Groups III-V are drawn to method for producing a vaccine to an antigen, however the specific steps involved in each method are different. The methods steps for Group I, II, III, IV, V, VI, VII, and VIII are all different and therefore lack the same technical feature. The methods (Groups I-VIII), chimeric proteins (Group IX), and covalent conjugates (Group X) do not share the same technical feature. The conjugates comprise cell wall proteins conjugated to an antigen or hapten or spacers, which is not the same as the chimeric protein.



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<p>(51) International Patent Classification <sup>6</sup> : G01N 33/53, 33/569, 33/536, 33/543, 33/566, 33/549, 33/531, C12N 1/20, 15/00, 15/63, 1/14, 15/09, 15/70, 15/74, A61K 38/00, C07K 2/00, 4/00, 5/00, 7/00, 16/00, 17/00, C12P 21/08, C07H 21/00, 21/02, 21/04</p>	<p>A1</p>	<p>(11) International Publication Number: <b>WO 97/08553</b>  (43) International Publication Date: 6 March 1997 (06.03.97)</p>
<p>(21) International Application Number: PCT/US96/14154 (22) International Filing Date: 22 August 1996 (22.08.96)  (30) Priority Data: 60/002,615 22 August 1995 (22.08.95) US 08/701,132 21 August 1996 (21.08.96) US  (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 22nd floor, 300 Lakeside Drive, Oakland, CA 94612-3550 (US).  (72) Inventors: SCHNEEWIND, Olaf; 11050 Strathmore Drive #422, Los Angeles, CA 90024 (US). BABA, Tadashi; 945 Gayley #306, Los Angeles, CA 90024 (US).  (74) Agent: FARBER, Michael, B.; Merchant, Gould, Smith, Edell, Welter &amp; Schmidt, Suite 400, 11150 Santa Monica Boulevard, Los Angeles, CA 90025 (US).</p>	<p>(81) Designated States: AU, BR, CA, IL, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report.</p>	
<p>(54) Title: TARGETING OF PROTEINS TO THE CELL WALL OF GRAM-POSITIVE BACTERIA  (57) Abstract  A method of stable noncovalent display of proteins, peptides, or compounds covalently linked to proteins or peptides on the surface of Gram-positive bacteria provides advantages over phage display. One embodiment of the present invention comprises a method for noncovalent protein targeting, comprising the steps of: (1) cloning a nucleic acid segment encoding a chimeric protein into a Gram-positive bacterium to generate a cloned chimeric protein including therein a carboxyl-terminal cell-wall targeting signal (2) growing the bacterium into which the nucleic acid segment has been cloned to express the chimeric protein to generate a chimeric protein including therein a carboxyl-terminal cell-wall targeting signal; and (3) binding the expressed chimeric protein noncovalently and stably to the cell-wall via the carboxyl-terminal cell-wall targeting signal so that the chimeric protein is displayed on the surface on the Gram-positive bacterium in such a way that the protein is accessible to a ligand. Alternatively, the chimeric protein can be produced by expression in another expression system and contacted with the Gram-positive bacterium.</p>		

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